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- The specification, claims and drawings as filed with the application on the filing date indicated above.

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METAL ION CHELATES BINDING MEMBRANE PROTEINS

FIELD OF INVENTION

The present invention relates to methods of identifying membrane proteins which bind metal ions, mapping metal ion binding sites of membrane proteins, screening for compounds binding to the metal ion binding sites of membrane proteins, and designing compound libraries and improving the binding affinity of compounds binding to the metal ion binding sites of membrane proteins.

BACKGROUND OF THE INVENTION

Membrane proteins constitute a numerous and varied group of proteins whose function is, *inter alia*, to mediate intercellular communication and communication between the cell exterior and the interior by transducing chemical signals across cell membranes. Membrane proteins are for instance receptors and ion channels to which specific chemical messengers termed ligands are bound resulting in the generation of a signal giving rise to a specific intracellular response (this process is known as signal transduction). Most membrane proteins are anchored in the cell membrane by a sequence of amino acid residues which are predominantly hydrophobic to form hydrophobic interactions with the lipid bilayer of the cell membrane. Such membrane proteins are also known as integral membrane proteins. In most cases, the integral membrane proteins extend through the cell membrane into the interior of the cell, thus comprising an extracellular domain, one or more transmembrane domains and an intracellular domain.

Many membrane proteins have become important targets for drug development in that irregularities in cellular signalling, that is, either increased or decreased signal transduction, from these proteins have been found to play a role in the development of a wide variety of diseases. Recent drug development has therefore focused on screening for compounds which are capable of either upregulating or downregulating the activity of the membrane protein, as required. Screening has usually been performed in a "shot-gun" fashion by setting up an assay for screening large numbers of compounds, e.g. compounds in combinatorial libraries, to identify compounds with the desired activity. Optimization of the hits from such screening procedures has been quite cumbersome and resource-demanding, involving procedures such as described by E. Sun and F.E. Cohen,

Gene 1993 137(1),127-32, or J. Kuhlmann, *Int J Clin Pharmacol Ther.* 1999 37(12),575-83. A major disadvantage of the classical drug discovery process is that it is difficult to identify active compounds with sufficient selectivity and specificity for a given target membrane protein and the desired pharmaco-kinetic properties.

For ligand optimization purposes, it is often an advantage to elucidate the ligand binding sites of various membrane proteins. The information obtained from these various procedures may be used to acquire a better understanding of ligand binding to membrane proteins involved in dysfunction or disease which is important in the design of new selective drugs towards the target in question.

Thus, site-directed mutagenesis is used to eliminate a ligand binding site or part of a ligand binding site by substitution of selected amino acid residues with other residues, e.g. alanine. Only a few cases have been presented where ligand binding sites have been thoroughly investigated by an extensive and systematic mutational analysis of all possible residues in a given area (e.g. the β-adrenergic receptor, Strader et al., *FASEB J. 3*, 1989, pp. 1825-1832; Strader et al, *J. Biol. Chem. 266*(1), 1991, pp. 5-8). A general problem of the site-directed mutagenesis method is that it is not clear whether the substitution of a residue affects the binding of a ligand directly (i.e. the residue is directly involved in ligand binding) or indirectly (i.e. the residue is only involved in the structure of the receptor).

Another problem of Ala substitution is false negative results because the procedure basically creates another "hole" in the presumed binding pocket through removal of the side chain on the residue replaced by Ala. The effect of Ala substitution is highly dependent on the relative contribution to the binding energy of the replaced residue. An alternative to Ala substitution is to introduce a larger side chain, e.g. Trp, in a presumed binding pocket as described by Holst et al., *Mol Pharmacol. 53*(1), 1998, pp. 166-175. This could cause many other problems for the ligand by impairing the interaction not only with the mutated residue but presumably also with neighbouring residues due to the incongruence that would be created in a larger part of the interface.

Exchange of all polar hydrogen-bond forming residues in the outer part of the receptor has been used to identify major interaction sites for non-peptide antagonists in the tachykinin receptors (Fong et al., *Nature 362*, 1993, pp. 350-353).

Conserved residu s and non-conserved residues between homologous receptors may reveal useful information in the case where particular residues are of interest. For instance, for investigation of binding interactions of a positively charged ligand to acidic residues, all conserved Asp and Glu residues can be substituted with Asn or Gln in the event that both receptors bind the same ligand. On the other hand, all non-conserved Asp and Glu residues can be substituted in the event that only one of the receptors bind the ligand.

Chimeric receptor constructs can be made to locate domains involved in ligand binding. The method was first described for G-protein coupled receptors by Kobilka et al, *Science 240*, 1988, pp. 1310-1316. By combining a non-ligand binding receptor with a ligand-binding receptor in different ways, useful information can be obtained to pinpoint the area of binding. Most often chimeric receptors are constructed between receptors with a certain degree of homology (e.g. NK-1 and NK-2, Gether et al., *Proc. Natl. Acad. Sci. USA 90*(13), 1993, 6194-6198), or the same receptor from different species (e.g. hCXR4 and rCXR4, Labrosse et al., *J. Virol. 72*(8), 1998, 6381-6388).

Photoaffinity labelling has been proven to be a useful tool in identifying domains of receptors involved in ligand binding (Dohlman et al., *Ann. Rev. Biochem. 60*, 1991, pp. 653-688). A photoreactive group is attached or built into the ligand. After binding, the ligand-receptor complex is exposed to UV light, resulting in crosslinking of the ligand to the receptor. Finally the complex is digested with proteases and the ligand-binding part of the receptor can be identified.

Structure-activity relationships (SAR) can provide a great deal of information regarding the nature of ligand-receptor interactions, but no information about the location of the binding site is provided. A number of closely related chemical structures are used to direct the orientation of the ligand within the putative binding cavity and to determine what part of the ligand is involved in binding to the receptor. This technique has its limitations due to the fact that changing the structure of the ligand may result in a change in the binding site of the receptor. The information obtained from the SAR can be used to build a pharmacophore model which contains information as to the parts of the ligand that are important for the binding and activity of the ligand. SAR in combination with site-directed mutagenesis, e.g. testing different ligands on different receptor mutants, may reveal interesting structural information, e.g. distance constraints within the receptor.

Finally, determination of the crystal structure by X-ray crystallography of three-dimensional crystals provides very high resolution structures and consequently very high quality information about the various structural features of the membrane protein. However, crystal structures are very difficult and consequently very expensive to provide, and the crystal structure has therefore only been elucidated for a few of the vast number of membrane proteins of potential interest as drug discovery targets.

A wide spectrum of ligands have been identified for the various families of integral membrane proteins. The types of chemical messengers acting through membrane proteins include ions, amino acids, monoamines, lipids, purines, neuropeptides, peptide hormones, chemokines, glycoprotein hormones and proteases. It has been found that several integral membrane proteins include binding sites for metal ions. The coordination of metal ions to metal ion binding sites is well characterized in numerous high-resolution X-ray structures of soluble proteins; for example, distances from the chelating atoms to the metal ion as well as the preferred conformation of the chelating side chains are known (e.g. J.P. Glusker, *Adv. Protein Chem. 42*, 1991, pp. 3-76; P. Chakrabarty, *Protein Eng. 4*, 1990, pp. 57-63; R. Jerigan et al., *Curr. Opin. Struct. Biol. 4*, 1994, pp. 256-263). Hence, characterizing a metal ion binding site in a membrane protein using, for example, molecular models and site directed mutagenesis yields information about the structure of the membrane protein and importantly where the "ligand" (metal ion) bind (e.g. Elling et al. *Fold. Des.* 2(4), 1997, pp. S76-80).

Engineering artificial metal ion binding sites in membrane proteins has also been employed to explore the structure of the protein. If side chains of amino acid residues participating in metal ion binding are known, it imposes a distance constraint on the protein structure which can be used in the interpretation of unknown protein structures (C.E. Elling and T.W. Schwartz, *EMBO J. 15*(22), 1996, pp. 6213-6219; C.E. Elling et al., *Fold. Des. 2*(4), 1997, pp. S76-80). C.E. Elling et al., *Nature 374*, 1995, pp. 74-77, have reported the conversion of an antagonist binding site in the tachykinin NK-1 receptor to a metal ion binding site. The metal ion binding sites created so far have all been antagonistic, i.e. generating inactive membrane proteins, but recently the generation of an activating metal ion binding site has been reported for the β_2-adrenergic receptor (C.E. Elling et al, *PNAS 96*, 1999, pp. 12322-12327).

SUMMARY OF THE INVENTION

The present invention utilises the finding that certain membrane proteins naturally have binding sites for metal ions. It is believed that the presence of metal ion binding sites is more general in membrane proteins than currently known, and the identification of metal ion binding sites in a substantial number of membrane proteins is expected to lead to not only a more extensive knowledge of the structure of these proteins, but also a more rational approach to the discovery of compounds acting on these targets based on the elucidation of the binding site and binding mode of metal ion containing compounds acting as ligands of the target proteins.

In the course of research leading to the present invention, the inventors have found that certain small organic molecules which bind metal ions (i.e. metal ion chelators) are also able to bind to metal ion binding sites in integral membrane proteins, in particular receptors, in such a way that the metal ion acts as a bridge between the small organic molecule and the receptor. These compounds bind with affinities corresponding to the binding affinities of most lead compounds discovered by simple screening of large chemical libraries. Importantly, the present invention has made it possible to identify and localise the exact binding site and binding mode of such metal ion chelates used as test compounds, contrary to what has generally been known in the art about the binding of test compounds or even high-affinity drugs. Based on the identification of the binding site of the test compounds, using for example site directed mutagenesis, molecular models of the receptors and techniques as described above, a rational approach may be taken to the chemical optimisation of the test compounds. Thus, relatively small chemical libraries may be made, the compounds in which may be designed to interact with amino acid residues of the binding site of the membrane protein in question. Compounds that exhibit a high affinity binding to the membrane protein may then be selected for further optimization.

Accordingly, the present invention relates to a method of identifying a membrane protein comprising a metal ion binding site, the method comprising contacting a membrane protein with a test compound which comprises a moiety including at least one heteroatom for chelating a metal ion and further comprising a metal ion chelated thereto for binding to at least one amino acid residue of said membrane protein, under conditions permitting binding of the test compound to the membrane protein, and determining any binding of said test compound to said membrane protein.

In another aspect, the invention relates to a method of mapping a metal ion binding site of a membrane protein, the method comprising

(a) contacting membrane protein with a test compound which comprises a moiety including at least one heteroatom for chelating a metal ion and further comprising a metal ion chelated thereto for binding to at least one amino acid residue of said membrane protein, under conditions permitting binding of the test compound to the membrane protein, and determining any binding of said test compound to said membrane protein, and

(b) determining, based on the primary structure of the specific membrane protein in question and the generic three-dimensional model of the class of membrane proteins to which the specific membrane protein of step (a) belongs, at least one metal ion binding amino acid residue located in the membrane protein to identify the metal ion binding site of said membrane protein.

In a further aspect, the invention relates to a method of screening for compounds capable of binding to a metal ion binding site of a membrane protein, the method comprising contacting a membrane protein with one or more test compounds which comprise a moiety including at least one heteroatom for chelating a metal ion and further comprising a metal ion chelated thereto for binding to at least one amino acid residue of said membrane protein, under conditions permitting binding of the test compound or compounds to the membrane protein, and determining any binding of said test compound or compounds to said membrane protein.

In a still further aspect, the invention relates to method of designing a compound library capable of binding to the metal ion binding site of a given membrane protein, the method comprising

(a) selecting a heteroalkyl or heterocyclyl metal ion chelate with a binding affinity to a given membrane protein of 50 μM or better as identified by the screening method indicated above,

(b) mapping the site of the membrane protein to which the chelate binds using the method indicated above,

(c) determining at least one amino acid residue potentially involved in interaction with at least one functional group of the chelate,

(d) providing th chelate with one or more functional groups of a nature and in a position optimised for interaction with said amino acid residue to generat a library of ch late derivatives,

(e) screening the derivatives of step (d) by the method indicated above, and

(f) optionally repeating any one or a combination of two or more of steps (a)-(e) one or more times to generate heteroalkyl or heterocyclyl metal ion chelating compounds with an improved binding affinity for the membrane protein.

In a still further aspect, the invention relates to a method of improving the binding affinity of a heteroalkyl or heterocyclyl metal ion chelate to a metal ion binding membrane protein, the method comprising

(a) selecting a heteroalkyl or heterocyclyl metal ion chelate with a binding affinity to a given membrane protein of 50 μM or better as identified by the screening method indicated above,

(b) mapping the site of the membrane protein to which the chelate binds using the method indicated above,

(c) determining at least one amino acid residue (potentially) involved in interaction with at least one functional group of the chelate,

(d) providing the chelate with one or more functional groups of a nature and in a position optimised for interaction with said amino acid residue to generate a library of chelate derivatives,

(e) screening the derivatives of step (d) by the method indicated above, and

(f) optionally repeating any one or a combination of two or more of steps (a)-(e) one or more times to generate heteroalkyl or heterocyclyl metal ion chelating compound library with an improved binding affinity for the membrane protein.

DETAILED DESCRIPTION OF THE INVENTION

In the following description and claims, the following terms shall be defined as indicated below.

A "test compound" is intended to indicate any drug, substance, compound or molecule with potential biological activity.

A "metal ion chelator" is intended to indicate a compound capable of forming a complex with a metal ion.

A "metal ion chelate" is intended to indicate a complex of a metal ion chelator and a metal ion.

A "ligand" is intended to include any substance that either inhibits or stimulates the activity of the membrane protein or that compete for the receptor in a binding assay. An "agonist" is defined as a ligand increasing the functional activity of a membrane protein (e.g. signal transduction though a receptor). An "antagonist" is defined as a ligand decreasing the functional activity of a membrane protein either by inhibiting the action of an agonist or by its own intrinsic activity. An "inverse agonist" (also termed "negative antagonist") is defined as a ligand decreasing the basal functional activity of a membrane protein.

A "metal ion binding site" is intended to indicate an amino acid residue of a membrane protein which comprises an atom capable of complexing with a metal ion. Such an atom will typically be a heteroatom, in particular N or S.

A "membrane protein" or "integral membrane protein" is intended to include any protein anchored in a cell membrane and mediating cellular signalling from the cell exterior to the cell interior. Important classes of membrane proteins include receptors such as tyrosine kinase receptors, G-protein coupled receptors, adhesion molecules, ligand- or voltage-gated ion channels, or enzymes. The term is intended to include membrane proteins whose function is not known, such as orphan receptors. In recent years, largely as part of the human genome project, large numbers of receptor-like proteins have been cloned and sequenced, but their function is as yet not known. The present invention may be of use in elucidating the function of the presumed receptor proteins by making it possible to identify and map metal ion binding sites in the proteins by means of metal ion chelates binding to the proteins.

"Signal transduction" is defined as the process by which extracellular information is communicated to a cell by a pathway initiated by binding of a ligand to a membrane protein, leading to a series of conformational changes resulting in a physiological change in the cell in the form of a cellular signal.

The term "better" when used to indicate the concentration of t st compound required to obtain a detectable binding to the membrane protein is intended to indicate a concentration below about μM. The concentration of the test compound required for binding to the membrane protein should preferably be lower than 10 μM, more particularly 1 μM or lower, and more preferably in the nanomolar range such as 500 nM or lower.

More specifically, the method of identifying a membrane protein comprising a metal ion binding site may comprise the following steps:

(a) selecting a nucleotide sequence suspected of coding for a membrane protein and deducing the amino acid sequence thereof,
(b) expressing said nucleotide sequence in a suitable host cell,
(c) contacting said cell or a portion thereof including the expressed membrane protein with a test compound which comprises a moiety including at least one heteroatom for chelating a metal ion and further comprising a metal ion chelated thereto for binding to at least one amino acid residue of said membrane protein, under conditions permitting binding of the test compound to the membrane protein, and determining any binding of said test compound to said membrane protein, and
(d) determining, based on the generic three-dimensional model of the class of membrane proteins to which the membrane protein or suspected membrane protein belongs, at least one metal ion binding amino acid residue located in said membrane protein to locate the metal ion binding site of said membrane protein.

As used herein the term "nucleotide sequence" is intended to indicate any nucleic acid molecule of cDNA, genomic DNA, synthetic DNA or RNA origin. The nucleotide sequence may be single- or double-stranded, and which may be based on a complete or partial naturally occurring nucleotide sequence encoding a membrane protein of interest to the present invention. The nucleotide sequence may optionally contain other nucleic acid segments.

The nucleotide sequence may suitably be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the polypeptide by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2. Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

For the present purpose, the DNA sequence encoding the polyp ptide is pref rably of vertebrate origin, e.g. derived from a mammalian, preferably human genomic DNA or cDNA library.

The nucleotide sequence encoding the membrane protein may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and Caruthers, *Tetrahedron Letters 22* (1981), 1859-1869, or the method described by Matthes et al., *EMBO Journal 3* (1984), 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

Furthermore, the nucleotide sequence may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire nucleotide sequence, in accordance with standard techniques.

The nucleotide sequence may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or Saiki et al., *Science 239* (1988), 487-491.

The nucleotide sequence encoding the membrane protein may suitably be inserted in to a suitable vector which may be any vector which may conveniently be subjected to recombinant DNA procedures. The choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the nucleotide sequence encoding the membrane protein is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the nucleotide sequence coding for the mem-

brane protein. The promoter may be any DNA sequence which shows transcriptional activity in th host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the nucleotide sequence encoding the polypeptide of the invention in mammalian cells are the ubiquitin promoter (Wiborg et al., *EMBO J. 4* (1985), 755-759), the SV40 promoter (Subramani et al., *Mol. Cell Biol. 1* (1981), 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., *Science 222* (1983), 809-814) or the adenovirus 2 major late promoter.

An example of a suitable promoter for use in insect cells is the polyhedrin promoter (US 4,745,051; Vasuvedan et al., *FEBS Lett. 311*, (1992) 7-11), the P10 promoter (J.M. Vlak et al., *J. Gen. Virology 69*, 1988, pp. 765-776), the Autographa californica polyhedrosis virus basic protein promoter (EP 397 485), the baculovirus immediate early gene 1 promoter (US 5,155,037; US 5,162,222), or the baculovirus 39K delayed-early gene promoter (US 5,155,037; US 5,162,222).

Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., *J. Biol. Chem. 255* (1980), 12073-12080; Alber and Kawasaki, *J. Mol. Appl. Gen. 1* (1982), 419-434) or alcohol dehydrogenase genes (Young et al., in *Genetic Engineering of Microorganisms for Chemicals* (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4,599,311) or ADH2-4c(Russell et al., *Nature 304* (1983), 652-654) promoters.

The nucleotide sequence encoding the polypeptide of the invention may also, if necessary, be operably connected to a suitable terminator, such as the human growth hormone terminator Palmiter et al., op.cit.) or (for fungal hosts) the TPI1(Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) terminators. The vector may further comprise elements such as polyadenylation signals (e.g. from SV40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication.

When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and origin of replication.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, *Gene 40*, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate.

The procedures used to ligate the nucleotide sequences coding for the membrane protein, the promoter and optionally the terminator sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op.cit.).

The nucleotide sequence encoding the membrane protein introduced into the host cell may be either homologous or heterologous to the host in question. If homologous to the host cell, i.e. produced by the host cell in nature, it will typically be operably connected to another promoter sequence or, if applicable, another terminator sequence than in its natural environment. The term "homologous" is intended to include a nucleotide sequence encoding a polypeptide native to the host organism in question. The term "heterologous" is intended to include a nucleotide sequence not expressed by the host cell in nature. Thus, the nucleotide sequence may be from another organism, or it may be a synthetic sequence.

The host cell into which the nucleotide sequence or the recombinant vector is introduced may be any cell which is capable of producing the present membrane protein and includes yeast cells and higher eukaryotic cells such as insect or mammalian cells.

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650 and 1651), BHK (ATCC CRL 1632, ATCC CCL 10), CHL (ATCC CCL39), CHO (ATCC CCL 61) 293 (ATCC CRL 1573) or NIH/3T3 (ATCC CRL 1658) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, *J. Mol. Biol. 159* (1982), 601-621; Southern and Berg, *J. Mol. Appl. Genet. 1* (1982), 327-341; Loyter et al., *Proc. Natl. Acad. Sci. USA 79* (1982), 422-

426; Wigler et al., *Cell 14* (1978), 725; Corsaro and Pearson, *Somatic Cell Genetics 7* (1981), 603, Graham and van der Eb, *Virology 52* (1973), 456, Neumann et al., *EMBO J.* (1982), 841-845; and in accordance with standard techniques (cf. Celis et al., *Cell Biology: A Laboratory Handbook*, 2. Ed. Academic Press, ISBN 0-12-164726-9 and; Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2. Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

Examples of suitable yeast cells include cells of Saccharomyces spp. or Schizosaccharomyces spp., in particular strains of Saccharomyces cerevisiae or Saccharomyces kluyveri. Methods for transforming yeast cells with heterologous DNA and producing heterologous polypeptides therefrom are described, e.g. in US 4,599,311, US 4,931,373, US 4,870,008, 5,037,743, and US 4,845,075, all of which are hereby incorporated by reference. Transformed cells are selected by a phenotype determined by a selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient, e.g. leucine. A preferred vector for use in yeast is the POT1 vector disclosed in US 4,931,373. The DNA sequence encoding the polypeptide of the invention may be preceded by a signal sequence and optionally a leader sequence, e.g. as described above. Further examples of suitable yeast cells are strains of Kluyveromyces, such as K. lactis, Hansenula, e.g. H. polymorpha, or Pichia, e.g. P. pastoris (cf. Gleeson et al., *J. Gen. Microbiol. 132*, 1986, pp. 3459-3465; US 4,882,279).

Transformation of insect cells and expression of heterologous membrane protein therein may be performed as described in US 4,745,051; US 4,879,236; US 5,155,037; 5,162,222; EP 397,485) all of which are incorporated herein by reference. The insect cell line used as the host may suitably be a Lepidoptera cell line, such as Spodoptera frugiperda cells or Trichoplusia ni cells (cf. US 5,077,214). Culture conditions may suitably be as described in, for instance, WO 89/01029 or WO 89/01028, or any of the aforementioned references.

The transformed or transfected host cell described above is then cultured in a suitable nutrient medium under conditions permitting the expression of the membrane protein, after which the cell expressing the membrane protein is recovered from the culture and used in a suitable assay to determine the activity of the membrane protein in the presence of test compounds.

The medium used to culture th cells may be any conventional medium suitable for growing the host cells, such as minimal or compl x media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection).

The membrane protein used in the method of the invention is suitably an integral membrane protein, which is to say a membrane protein anchored in the cell membrane. The membrane protein is preferably of a type comprising at least one transmembrane domain. Interesting membrane proteins for the present purpose are found in classes comprising 1-14 transmembrane domains.

Thus, membrane proteins of interest comprising one transmembrane domain are receptors such as tyrosine kinase receptors, e.g. a growth factor receptor such as the growth hormone, insulin, epidermal growth factor, transforming growth factor, erythropoietin, granulocyte colony-stimulating factor, platelet-derived growth factor or nerve growth factor receptor (TrkA or TrkB).

Membrane proteins of interest comprising two transmembrane domains are, e.g., purinergic ion channels.

Membrane proteins of interest comprising 3, 4 or 5 transmembrane domains are, e.g., ligand-gated ion channels, such as nicotinic acetylcholine receptors, GABA receptors or glutamate receptors (NMDA or AMPA).

Membrane proteins of interest comprising 6 transmembrane domains are, e.g., voltage-gated ion channels, such as potassium, sodium, chloride or calcium channels.

Membrane proteins of interest comprising 7 transmembrane domains are, e.g., G-protein coupled receptors, such as the acetylcholine, adenosine, adrenoceptors, anaphylatoxin chemotactic receptor, angiotensin, bombesin (neuromedin), bradykinin, calcitonin, calcitonin gene related peptide, conopressin, corticotropin releasing factor, amylin, adrenomedullin, calcium, cannabinoid, chemokine, cholecystokinin, conopressin, corticotropin-releasing factor, dopamine, eicosanoid, endothelin, fMLP, $GABA_B$, galanin, gastrin, gastric inhibitory peptide, glucagon, glucagon-like, glutamate, glycoprotein hormone (e.g. FSH, LSH, TSH, LH), gonadotropin releasing hormone, growth hormone releasing hormone,

growth hormone secretagogue, histamine, 5-hydroxytryptamine, leukotriene, lysophospholipid, melanocortin, melatonin, neuropeptide Y, neurotensin, olfactory, opioid, opioid-like, opsin, orexin, oxytocin, parathyroid hormone/parathyroid hormone-related, P2Y, pheromone, platelet-activating factor, prostanoid, protease-activated, secretin, somatostatin, tachykinin, thrombin and protease activated, thyrotropin-releasing hormone, pituitary adenylate activating peptide, vasopressin, vasoactive intestinal peptide and viral encoded receptors; in particular galanin, P2Y, chemokine, melanocortin, bombesin, cannabinoid, lysophospholipid, fMLP, neuropeptide Y, tachykinin, dopamine, histamine, 5-hydroxytryptamine, histamine, mas-proto-oncogene, acetylcholine, oxytocin, herpes virus encoded, epstein-barr virus encoded, cytomegalovirus encoded and bradykinin receptors; preferably galanin receptor type 1, leukotriene B4 receptor, CXCR5, melanocortin-1 receptor, melanocortin-3 receptor, melanocortin-4 receptor, melanocortin-5 receptor, bombesin receptor subtype 3, cannabinoid receptor 1, cannabinoid receptor 2, EDG-2, EDG-4, FMLP-related receptor I, FMLP-related receptor-II, Y6 receptor, Y5 receptor, NK-1 receptor, NK-3 receptor, D2 receptor (short), D2 receptor (long), D4 receptor (D2C), duffy antigen, Q89609 herpes virus type 2, VU51_HSV6U, histamine H1 receptor, MAS proto-oncogene, muscarinic M1 receptor, muscarinic M2 receptor, muscarinic M3 receptor, muscarinic M5 receptor, oxytocin receptor, XCR1 receptor, EBI2 receptor, RDC1 receptor, O76000 receptor, O15218 receptor, GPR12 receptor or GPR3 receptor.

Membrane proteins of interest comprising 8, 10, 12 or 14 transmembrane domains are, e.g., transporter proteins, such as a GABA, monoamine or nucleoside transporter.

The membrane protein may also be a multidrug resistance protein, e.g. a P-glycoprotein, multidrug resistance associated protein, lung resistance related protein, drug resistance associated protein, breast cancer resistance protein, adenosine triphosphate-binding cassette protein, Bmr, QacA or EmrAB/TolC.

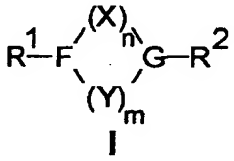
The membrane protein may also be a cell adhesion molecule, e.g. NCAM, VCAM or ICAM.

Furthermore, the membrane protein may be an enzyme such as adenylyl cyclase.

Test compounds which have been found to be useful in the present methods are typically compounds comprising a heteroalkyl or heterocyclyl moiety for chelating the metal ion.

The term "heteroalkyl" is understood to indicate a branched or straight-chain chemical entity of 1-15 carbon atoms containing a heteroatom. The term "heterocyclyl" is intend d to indicate a cyclic unsaturated, aromatic ("heteroaryl") or saturated ("heterocycloalkyl") group comprising at least one heteroatom. Preferred "heterocyclyl" groups comprise a 5- or 6-membered rings with 1-3 heteroatoms or fused 5- or 6-membered rings comprising 1-4 heteroatoms. The heteroatom is typically N, O or S. Examples of heteroaryl groups are indolyl, dihydroindolyl, thiophenyl, furanyl, benzofuranyl, pyridinyl, pyrimidinyl, quinolinyl, triazolyl, imidazolyl, thiazolyl, tetrazolyl and benzimidazolyl. The heterocycloalkyl group generally includes 3-20 carbon atoms and 1-4 heteroatoms.

Particularly useful test compounds are those of general formula I



I

wherein F is N, O or S and G is N, O or S;

and R^1 is alkyl, aryl, cycloalkyl, alkoxy, ester, heteroalkyl, heterocycloalkyl or heteroaryl group, optionally substituted with one or more substituents selected from halogen, nitro, cyano, amino, alkyl, alkoxy, carboxy, amide or sulfonamide;

R^2 is an alkyl, aryl, cycloalkyl, alkoxy, ester, heteroalkyl or heteroaryl group, optionally substituted with one or more substituents selected from halogen, nitro, cyano, amino, alkyl, alkoxy, carboxy, amide or sulfonamide;

R^1 and/or R^2 optionally forming a fused ring together with the heteroatom to which it is attached, or R^1 and/or R^2 optionally forming a fused ring together with the heteroatom to which it is attached and a carbon atom of X or Y;

X is $-CH_2-$, CH_2-CH_2-, $-CH_2-S-CH_2-$, $-CH_2-N-CH_2-$, $-CH=CH-CH=CH-$, $-CH_2-(Z)_a-(P)_c-(W)_b-CH_2-$, $-CH_2-O-CH_2-$, wherein

each of Z and W are independently C, S, O or N, and

P is -CH- or $-CH_2-$;

Y is $-CH_2-$, CH_2-CH_2-, $-CH_2-S-CH_2-$, $-CH_2-N-CH_2-$, $-CH=CH-CH=CH-$, $-CH_2-(Z)_a-(P)_c-(W)_b-CH_2-$, $-CH_2-O-CH_2-$, wherein

each of Z and W are independently C, S, O or N, and

P is -CH- or $-CH_2-$;

n is 0 or an integer of 1-5,

m is 0 or an integer of 1-5,
a is an integer of 1-3,
b is an integer of 1-3, and
c is an integer of 1-7.

In the present context, the term "alkyl" is intended to indicate a branched or straight-chain, saturated or unsaturated chemical group containing 1-10, preferably 1-8, in particular 1-6 carbon atoms, such as methyl, ethyl, propyl, isopropyl, sec. butyl, tert. butyl, pentyl, isopentyl, hexyl, isohexyl, heptyl etc.

The term "cycloalkyl" is intended to denote a cyclic, saturated alkyl group of 3-7 carbon atoms.

The term "aryl" is intended to denote an aromatic (unsaturated), typically 5- or 6-membered, ring which may be a single ring (e.g. phenyl) or fused with other 5- or 6-membered rings (e.g. naphthyl or anthracyl).

The term "alkoxy" is intended to indicate the group alkyl-O-.

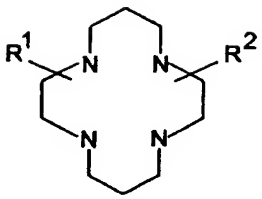
The term "amino" is intended to indicate the group -NR'R" where R' and R" are independently hydrogen, alkyl substituted alkyl, aryl, arylalkyl, substituted arylalkyl, heteroaryl, and substituted heteroaryl. In a primary amine group, both R' and R" are hydrogen, whereas in a secondary amino group, either but not both R' and R" is hydrogen.

The term "carboxy" is intended to indicate the group R'-COOH, where R' is as indicated above except hydrogen.

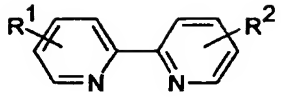
The term "ester" is intended to indicate the group COO-R', where R' is as indicated above except hydrogen.

Examples of halogen include fluorine, chlorine, bromine and iodine.

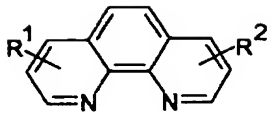
For the purpose of locating the presence of metal ion binding sites in membrane proteins, test compounds in which the heterocyclic moiety is selected from a compound of formula IIa, IIb or IIc



IIa



IIb

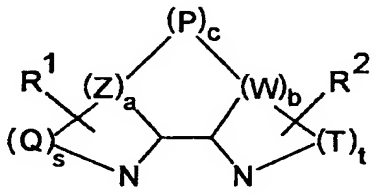


IIc

wherein R^1 and R^2 are as indicated above in formula I.

have been found to be particularly suitable.

For the purpose of mapping the metal ion binding site or for screening for compounds capable of binding to the metal ion binding site of the membrane protein, test compounds comprising a heterocyclic moiety of the general formula III

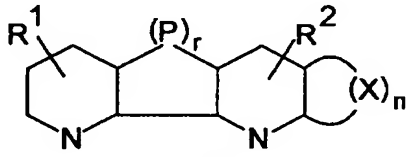


III

wherein R^1, R^2, Z, W, P, a, b and c are as indicated above, and

each of Q and T is independently -CH- or $-CH_2-$, s is an integer of 1-7, and t is an integer of 1-7, are believed to be particularly suitable.

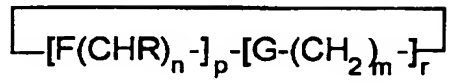
In particular, test compounds in which the heterocyclyl moiety has the general formula IV



IV

wherein R^1, R^2, P, X and n are as indicated above, r is an integer of 1-3 are believed to be useful for mapping and screening purposes.

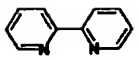
More preferred test compounds are those in which the heterocyclyl moiety has the g n-eral formula V

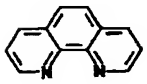


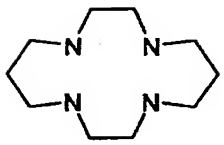
V

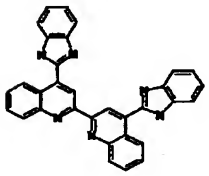
wherein F is N,O or S and G is N,O or S,
n is an integer from 1 to 5,
m is 0 or an integer from 1 to 5,
p is 0 or an integer from 1 to 8,
r is 0 or an integer from 1 to 8, and
R is alkyl, aryl, cycloalkyl, alkoxy, heteroalkyl or heteroaryl, optionally substituted with one or more substituents selected from halogen, nitro, cyano, amino, alkyl, alkoxy, carboxy, amide or sulfonamide.

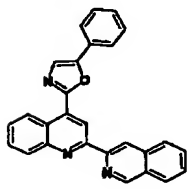
Examples of test compounds may be those in which the heterocyclic moiety is selected from a compound shown in Table 1:

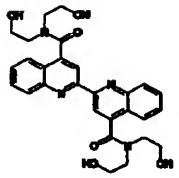
VII 

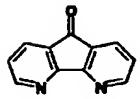
VIII 

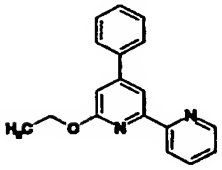
IX 

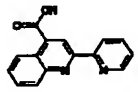
X 

XI 

XII 

XIII 

XIV 

XV 

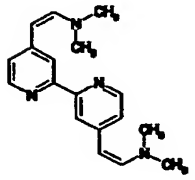
XVI 

Table I

As indicated above, the membrane proteins of interest to this invention comprise a metal ion binding site, i.e. one or more amino acid residues capable of binding ions. Amino acid residues which function as effective metal ion binding residues are typically those that contain electron donating atoms such as S, O or N. Alternatively, metal ion binding amino acid residues may be found among aromatic amino acids. Thus, preferred candidate amino acids for binding metal ions are those selected from Ser, Lys, Arg, Tyr, Thr, Trp, Phe, Asp, Glu, Asn, Gln, Cys and His, in particular Asp, Glu, Cys and His, preferably His.

Metal ions forming the complex with the heteroalkyl or heterocyclyl moiety in the test compounds may advantageously be selected from metal ions which have been tested for or are used for pharmaceutical purposes. Thus the metal ion is selected from the group consisting of rubidium, caesium, beryllium, magnesium, calcium, strontium, barium, scandium, yttrium, lutetium, titanium, zirconium, hafnium, vanadium, niobium, tantalum, chromium, molybdenum, tungsten, manganese, technetium, rhenium, iron, ruthenium, osmium, cobalt, rhodium, iridium, nickel, palladium, platinum, copper, silver, gold, zinc, cadmium, mercury, boron, aluminium, gallium, indium, thallium, silicon, germanium, tin, lead, arsenic, antimony, bismuth, tellurium, polonium, astatine, lanthanum, cerium, praseodymium, neodymium, promethium, samarium, europium, gadolinium, terbium, dysprosium, holmium, erbium, thulium, ytterbium, thorium and oxidation states and isotopes thereof; in particular rubidium, magnesium, calcium, strontium, barium, yttrium, lutetium, chromium, manganese, technetium, rhenium, iron, ruthenium, osmium, cobalt, rhodium, nickel, palladium, platinum, copper, silver, gold, zinc, aluminium, gallium, indium, thallium, germanium, tin, antimony, bismuth samarium, europium, gadolinium, terbium, thorium and oxidation states or isotopes thereof; preferably magnesium (II), calcium (II), manganese (II), iron (II) and (III), ruthenium (II) and (III), nickel (II), palladium (II), platinum (II), copper (II), zinc (II), samarium (III), europium (III), terbium (III) or isotopes thereof.

For the present purpose, a particularly favourable test compound is a Cu^{2+}-phenanthroline complex, a Zn^{2+}-phenanthroline complex, a Mg^{2+}-phenanthroline complex, a Ca^{2+}-phenanthroline complex, a Cu^{2+}-bipyridyl complex, a Zn^{2+}-bipyridyl complex, a Mg^{2+}-bipyridyl complex, a Ca^{2+}-bipyridyl complex, a Cu^{2+}-1,4,8,11-tetraazacyclotetradecane, a Zn^{2+}-1,4,8,11-tetraazacyclotetradecane, a Mg^{2+}-1,4,8,11-tetraazacyclotetradecane, a Ca^{2+}-1,4,8,11-tetraazacyclotetradecane.

The screening method of the present invention may include a further step of determining, based on the primary structure of the specific membrane protein in question and the generic three-dimensional model of the class of membrane proteins to which the specific membrane protein belongs, at least one metal ion binding amino acid residue located in the membrane protein to identify the metal ion binding site of said membrane protein.

The term "generic three-dimensional model" is intended to indicate a model of the membrane protein established on the basis of, e.g., X-ray crystallographic data of a membrane

protein of the same family, electron d nsity maps of the membrane protein generated by cryo-electronmicroscopic analysis of two-dim nsional membrane crystals (Baldwin, *EMBO J. 12*(4), 1993, pp. 1693-1703; Baldwin, *Curr. Opi. Cell. Biol. 6*, 1997, pp. 180-190; Herzyk et al. *J. Mol.Biol. 281*(4), 1998, pp. 741-754), knowledge of helical or other structures of the particular membrane protein, etc.

Such a determination may be performed using a generic three-dimensional model of the membrane protein showing the spatial arrangement of the amino acid residues defining the area of the metal ion binding site, site-directed mutagenesis of at least one amino acid residue potentially involved in interaction with said metal ion chelated to the test compound, followed by expression of the mutated membrane protein in a suitable cell, contacting said cell or a portion thereof including the expressed mutated membrane protein with the test compound, and determining any effect on binding in a competitive binding assay using a labelled ligand of the membrane protein, detection of any changes in signal transduction from the membrane protein or using a chelating agent which is in itself detectable or labelled with a detectable labelling agent. If an amino acid residue involved in interaction with the metal ion is mutated to one which is not, this may be detected as a decrease in binding or other activity.

Site-directed mutagenesis may be performed in accordance with standard techniques, e.g. as described in Ho et al., *Gene 77*, pp. 51-59; Horton et al., *Gene 77*, pp. 61-68; or "*Current Protocols in Molecular Biology*", Ausubel et al, John Wiley & Sons Inc., ISBN 0-471-50338-X & 0-471-50337-1.

Furthermore, the screening method of the invention may comprise a further step of determining at least one other amino acid residue of the membrane protein binding at least one functional group of the test compound other than the metal ion.

Such a determination is performed using site-directed mutagenesis of a least one amino acid residue potentially involved in interaction with said functional group of the test compound other than the metal ion, followed by expression of the mutated membrane protein in a suitable cell, contacting said cell or a portion thereof including the mutated membrane protein with the test compound, and determining any effect on binding in a competitive binding assay using a labelled ligand of the membrane protein, detection of any changes in signal transduction from the membrane protein or using a chelating agent which is in

itself detectable or lab lled with a d tectable labelling agent. If an amino acid residue involved in interaction with such a functional group of the t st compound is mutated to one which is not, this may be detected as a decrease in binding or other activity.

As indicated above, a considerable number of membrane proteins comprising metal ion binding sites are believed to be potential targets for the development of drugs for the treatment of a variety of disease states some of which will require a decrease of signal transduction, i.e. receptor activity, and others an increase of signal transduction for treatment.

Consequently, the present invention relates to a method of preferentially stabilising a membrane protein comprising a metal ion binding site in an active conformation, the method comprising contacting said membrane protein with an effective amount of a heteroalkyl or heterocyclyl metal ion chelate acting as an agonist, partial agonist or positive modulator of the membrane protein.

A "partial agonist" is a compound which can only activate a membrane protein to a certain level compared to a full agonist. A "positive modulator" is a compound which is not able in itself to stimulate the activity of the membrane protein directly, but is able to do so indirectly through its impact on an agonist of the membrane protein. Such compounds are also known as enhancers.

Furthermore, the invention relates to a method of preferentially stabilising a membrane protein comprising a metal ion binding site in an inactive conformation, the method comprising contacting said membrane protein with an effective amount of a heteroalkyl or heterocyclyl metal ion chelate acting as an antagonist, partial antagonist, inverse agonist or negative modulator of the membrane protein.

An "inverse agonist" is a compound which is able to decrease the intrinsic, constitutive activity of a membrane protein. A "negative modulator" is a compound which is not in itself capable of inactivating the membrane protein, but is able to do so indirectly through its impact on an antagonist or inverse agonist of the membrane protein.

Although pharmaceutical drugs are known which are composed of or include metal ions, it may, in some instances, be less desirable to include a metal ion in a compound to be

used as a drug. In a further embodiment of the invention, it is therefore envisaged to remove the metal ion binding moiety from the heteroalkyl or heterocyclyl chelate, substituting the metal ion by a functional group binding to an amino acid residue in the ligand-binding domain of the membrane protein so as to maintain/effect high affinity binding to the ligand-binding domain. Examples of such functional groups are electrophiles such as the ammonium ion, or hydrogen bond donor or acceptor groups such as carboxy, amino, amide, sulphonate etc.

DESCRIPTION OF PREFERRED EMBODIMENTS

7TM receptors overview - A particularly interesting class of membrane proteins for use in the present invention is 7 transmembrane domain receptors (7TM receptors), also known as G-protein coupled receptors (GPCRs). This family of receptors constitutes the largest super-family of proteins in the human body and a large number of current drugs are directed towards 7TM receptors, for example: antihistamines (for allergy and gastric ulcer), beta-blockers (for cardiovascular diseases), opioids (for pain), and angiotensin antagonists (for hypertension). These current drugs are directed against relatively few receptors, which have been known for many years. To date, several hundred 7TMs have been cloned and characterized, and the total number of different types of 7TMs in humans is presumed to be between 1 and 2.000. The spectrum of ligands acting through 7TMs include a wide variety of chemical messengers such as ions (e.g. calcium ions), amino acids (glutamate, γ-amino butyric acid), monoamines (serotonin, histamine, dopamine, adrenalin, noradrenalin, acetylcholine, cathecolamine, etc.), lipid messengers (prostaglandins, thromboxane, anandamide, etc.), purines (adenosine, ATP), neuropeptides (tachykinin, neuropeptide Y, enkephalins, cholecystokinin, vasoactive intestinal polypeptide, etc.), peptide hormones (angiotensin, bradykinin, glucagon, calcitonin, parathyroid hormone, etc.), chemokines (interleukin-8, RANTES, etc.), glycoprotein hormones (LH, FSH, TSH, choriogonadotropin, etc.) and proteases (thrombin). It is expected that a large number of the members of the 7TM superfamily of receptors will be suitable as drug targets. This notion is based on the fact that these receptors are involved in controlling major parts of the chemical transmission of signals between cells both in the endocrine and the paracrine system in the body as well as within the nervous system.

7TM receptor signalling - In 7TMs, binding of the chemical messenger to the receptor leads to the association of an intracellular G-protein which, in turn, is linked to a secon-

dary messenger pathway. The G-protein consists of thre subunits, an α subunit that binds and hydrolyses GTP, a β subunit and a γ subunit. When GDP is bound, the α subunit associates with the βγ subunit to form an inactive heterotrimer that binds to the receptor. When the receptor is activated, a signal is transduced by a changed receptor conformation that activates the G-protein. This leads to the exchange of GDP for GTP on the α subunit, which subsequently dissociates from the receptor and the βγ dimer, and activates downstream second messenger systems (e.g. adenylyl cyclase). The α subunit will activate the effector system until its intrinsic GTPase activity hydrolyses the bound GTP to GDP, thereby inactivating the α subunit. The βγ subunit increases the affinity of the α subunit for GDP but may also be directly involved in intracellular signalling events.

Ligand-binding sites in general - Mutational analysis of 7TMs has demonstrated that functionally similar but chemically very different types of ligands apparently can bind in several different ways and still lead to the same function. Thus monoamine agonists appear to bind in a pocket relatively deep between TM-III, TM-V and TM-VI, while peptide agonists mainly appear to bind to the exterior parts of the receptors and the extracellular ends of the TMs (Strader et al., (1991) *J.Biol.Chem.* **266**, 5-8; Strader et al., (1994) *Ann.Rev.Biochem.* **63**, 101-132; Schwartz et al. *Curr. Pharm. Design.* (1995), **1**, 325-342). Moreover, ligands can be developed independent on the chemical nature of the endogenous ligand, for example non-peptide agonists or antagonists for peptide receptors. Such non-peptide antagonists for peptide receptors often bind at different sites from the peptide agonists of the receptors. For instance, non-peptide antagonists may bind in the pocket between TM-III, TM-V, TM-VI and TM-VII corresponding to the site where agonists and antagonists for monoamine receptors bind. It has been found that in the substance P receptor, when the binding site for a non-peptide antagonist has been exchanged for a metal ion binding site through introduction of His residues, no effect on agonist binding was observed (Elling et al., (1995) *Nature* **374**, 74-77; Elling et al., (1996) *EMBO J.* **15**, 6213-6219). It is believed that the non-peptide antagonist and the zinc ions act as antagonists by selecting and stabilizing an inactive conformation of the receptor, that prevents the binding and action of the agonist. This, illustrates that drugs can be developed totally independent on knowledge of the endogenous ligand, since there need not be any overlap in their binding sites.

Generic numbering system for 7TMs - There are several families of 7TM receptors, which betwe n each oth r are very div rg nt in respect of amino acid sequence. The

largest family of 7TM receptors is composed of the rhodopsin-like receptors, which are named after the light-sensing molecule from our eye. Besides sharing an overall transmembrane domain composed of a seven helical bundle, many 7TMs also share other structural features such as the presence of a disulfide bridge between the top of TM-III and the middle of extracellular loop 3. Within the many hundred members of the rhodopsin-like receptor family a number of residues are furthermore conserved - especially within each of the transmembrane segments; although only a single residue appear to be totally conserved in all signalling 7TM rhodopsin-like receptors, i.e. an Arg at the intracellular end of TM-III (ArgIII:26 – see below concerning the generic numbering nomenclature). Due to differences in length of especially the N-terminal segment, residues located at corresponding positions in different 7TM receptors are numbered differently in different receptors. However, based on the conserved key-residues in each TM, a generic numbering system has been suggested (JM Baldwin, *EMBO J.* **12(4)**, 1993, pp. 1693-1703; TW Schwartz, *Curr. Opin. Biotech.* **5**, 1994, pp. 434-444), which is used here. In Fig. 1, a schematic depiction of the structure of rhodopsin-like 7TMs is shown with one or two conserved, key residues highlighted in each TM: AsnI:18; AspII:10; CysIII:01 and ArgIII:26; TrpIV:10; ProV:16; ProVI:15; ProVII:17. In relation to the present invention it is important, that residues involved in for example metal-ion binding sites can be described in this generic numbering system. For example, a tri-dentate metal-ion site constructed in the tachykinin NK1 receptor (Elling et al., (1995) *Nature* **374**, 74-77) and subsequently transferred to the kappa-opioid receptor (Thirstrup et al., (1996) *J.Biol.Chem.* **271,** 7875-7878) and to the viral chemokine receptor ORF74 (Rosenkilde et al., *J. Biol. Chem.* 1999 Jan 8; 274(2), 956-61) can be described to be located between residues V:01, V:05, and VI:24 in all of these receptors although the specific numbering of the residues is very different in each of the receptors. It is only in the rhodopsin-like receptor family that a generic numbering system has been established; however, it should be noted that although the sequence identity between the different families of 7TM receptors is very low it is believed that they may share a more-or-less common seven helical bundle structure. Thus, all the techniques described in the present invention can be applied to the other families of 7TM receptors with minor modifications.

Metal-ion sites in 7TMs - Naturally occurring metal-ion sites have as yet only been described in two 7TM receptors, the tachykinin NK3 receptor (Rosenkilde et al., *FEBS Lett.* 1998 Nov 13; 439 (1-2), 35-40) and the galanin receptor (Kask et al., *EMBO J.* 1996 Jan 15; 15(2), 236). In the NK3 receptor Zn2+ was shown to act as an enhancer (positive

modulator) for agonist binding and action without itself being an agonist. Through mutagenesis the metal ion binding site was mapped to residues V:01 and V:05 at the extracellular end of TM-V (Rosenkilde et al., *FEBS Lett.* 1998 Nov 13; 439(1-2), 35-40). In the galanin receptor zinc was shown to act as an antagonist for galanin binding, but the site was not characterized further (Kask et al., *EMBO J.* 1996 Jan 15; 15(2), 236). In contrast much work has been done on building up artificial metal-ion sites in 7TM receptors (Elling et al., *Nature.* 1995 Mar 2; 374(6517), 74-7; Elling et al., *EMBO J.* 1996 Nov 15; 15(22), 6213-9; Elling et al., *Fold Des.* 1997; 2(4), S76-80; Elling et al., *Proc Natl Acad Sci USA* 1999 Oct 26; 96(22), 12322-7; Sheikh et al., *Nature.* 1996 Sep 26; 383(6598), 347-50). Based on this protein engineering work and on mutational analysis of ligand-binding sites as such at multiple locations in a number of wild-type 7TM receptors (Schwartz, T.W. (1994) *Curr.Opin.Biotech.* **5**, 434-444, Schwartz et al., *Curr. Pharm. Design*, 1995, **1**, 325-342) much experience has been gathered in relation to where metal-ion sites can be located in the 7TM receptor structure as such. It is this knowledge which can be applied to wild-type, natural 7TM receptors to predict the occurrence of metal ion binding sites which have not previously been noted. Based on sequence analysis and molecular models it is found that such sites surprisingly occur in multiple 7TM receptors. Only few of these sites are probably addressed physiologically by free metal ions, for example when a receptor is expressed in brain regions where extracellular zinc concentrations may vary around 10^{-5} molar. Probably, many of the other previously unnoticed metal-ion "sites" may just be a reflection of the fact that polar, metal ion binding amino acid residues (for example: His, Cys, Asp etc.) are frequently used by nature to face the water-exposed main ligand-binding crevice of 7TM receptors. These residues will in the current invention be used as initial attachment sites for metal-ion chelating test compounds in the drug discovery process. It has recently been demonstrated that metal ion chelators could bind in artificially engineered metal ion binding sites with high affinity (Elling et al., *Proc Natl Acad Sci USA* 1999 Oct 26; 96(22), 12322-7). In relation to the present invention, the work on the engineered metal ion binding sites can be viewed as an indirect demonstration of the general principle – however, performed by "reverse chemistry". That is, the metal ion binding site in the beta2-adrenergic receptor occurs in the mutated form of the receptor as opposed to the wild-type and the mutations are used to "build up" the metal ion binding site and not to "map it". To illustrate this further, unpublished sequence analysis shows that the activating metal ion binding site between AspIII:08 and HisVII:06, which was built artificially into the beta2-adrenergic receptor occurs naturally in the muscarinic M3 receptor and in the dopaminergic D4 receptor. Whether the III:08 to VII:06 site in these receptors in response to

free metal ions or various metal ion chelates is activating or inactivating r mains to be shown experimentally.

Metal ion chelates can bind in naturally occurring metal ion binding sites in 7TM drug targets – The galanin R1 receptor is a potential drug target. Galanin is a 29-30 residue neuropeptide widely distributed throughout the peripheral and central nervous systems and is involved as a regulatory mechanism in many physiological functions and is therefore highly interesting to the pharmaceutical industry (Langel and Bartfai, *op. cit.*). Its physiological effects are mediated through distinct galanin receptor subtypes (Gundlach and Burazin, *Ann. N.Y. Acad. Sci. 863*, 1998, pp. 241-251). The receptor cDNA for the human galanin receptor 1 (hGalR1) encodes a rhodopsin-like 7TM receptor of 349 amino acids (Habert-Ortoli et al., *Proc. Natl. Acad. Sci. USA 91*(21), 1994, pp. 9780-9783) (Fig. 2). The cloned hGalR1, expressed in COS cells, binds [^{125}I]galanin with high affinity (0.1-1 nM) (Habert-Ortoli et al., *op. cit.*). hGAlR1 was further shown to reduce forskolin-stimulated cAMP when stimulated with galanin peptides of high potency (0.1-1 nM). The GalR1-dependent reduction of cAMP was blocked by pertussis toxin, supporting the involvement of Gi-type G-protein (Parker et al., *Brain Res. Mol. Brain Res. 34*(2), 1995, pp. 179-189). Over the last few years, a wealth of data derived from behavioral, neurobiologic, and neuropathologic studies of patients with Alzheimer's disease have suggested that galanin plays crucial role in the modulation of cholinergic basal forebrain neurons (Crawley, *Life Sci. 58*(24), 1996, pp. 2185-2199). These neurons provide the major cholinergic innervation of the cortex and hippocampus (Mesulam et al., *J. Comp. Neurol. 214*(2), 1983, pp. 170-197) and are associated with cognitive function (Bartus et al., *Science 217*, 1982, pp. 408-414). In Alzheimer's disease these neurons undergo extensive degeneration that correlates with the duration of the disease and the degree of cognitive impairment (Wilcock et al., *J. Neurol. Sci. 57*(2-3), 1982, pp. 407-417). It has been suggested that overexpression of galanin may downregulate the function of remaining cholinergic basal forebrain neurons, further intensifying cholinergic dysfunction in Alzheimer's disease (Chan-Palay, *J. Comp. Neurol. 273*(4), 1988, 543-557). These observations suggest that the development of a potent galanin antagonist would be a useful step towards successful treatment of Alzheimer's disease. Current approved drug treatment for Alzheimer's disease consists of anticholinesterase agents that act by increasing the availability of synaptic acetylcholine. This results in an increased firing rate of remaining cholinergic neurons (Davis et al., *N. Eng. J. Med. 327*(18), 1992, pp. 1253-1259). It is thought that this elevated firing rate increases the release of galanin (Mufson et al., *Ann. N.Y.*

Acad. Sci. 863, 1998, pp. 291-304). However the problem is that galanin acts as an inhibitory modulator on the already depleted presynaptic release of acetylcholine (Chan-Palay, *op. cit.*). This negative feedback loop eventually leads to burnout of the remaining cholinergic neurons. Antagonists towards the galanin receptor could therefore enhance the cholinergic transmission by reducing the inhibitory influence of galanin on the cholinergic neuron firing rate. Using cell lines expressing the cloned galanin receptors, several large scale, high throughput screening programs are currently being carried out at pharmaceutical companies in search of low molecular weight galanin receptor ligands (Langel and Bartfai, *op. cit.*). So far only one patent application has been published, showing a non-peptide ligand of moderate affinity ($IC_{50} \approx 1$ µM).

Kask et al. (1996, *op. cit.*) have in a single experiment shown that Zn^{2+} can displace [^{125}I]galanin in a concentration-dependent manner from the GalR1 receptor, indicating that the human galanin receptor could possess a Zn^{2+} binding site. Several potential metal-binding residues are found in the major binding crevice of the galanin receptor (Figure 2). We find that Zn^{2+} do bind to the human galanin receptor with an affinity, IC_{50} of 74 µM. As shown in Fig. 3, free Cu^{2+} also binds to the galanin receptor and with an even higher affinity (IC_{50} = 26 µM) whereas phenanthroline does not bind to the wild-type galanin receptor. However, the complex of Zn^{2+} or Cu^{2+} with phenanthroline compete with [^{125}I]galanin binding for the receptor with an even higher affinity than the free metal-ions (Fig. 3). The high equilibrium constant of, for example Cu^{2+}-phenanthroline, 10^{21}M (Smith et al., *Critical Stability Constants of Metal Complexes Database 46*, 1993: NIST Database 46) results in an very low concentration of free Cu^{2+} ([Cu^{2+}]< 1 nM). Thus, the effect seen in Fig. 3 must caused by the metal chelates as such and not by the free ions. It is suggested that the phenanthroline metal ion chelate acts as a metal ion-guided ligand, in which the metal ion bridges the binding of phenanthroline to the galanin receptor.

Cu^{2+}-phenanthroline has recently been shown to bind to an engineered metal binding site in the β_2-adrenergic receptor (Elling et al., *Proc Natl Acad Sci USA* 1999 Oct 26; 96(22), 12322-7). It has been demonstrated that Cu^{2+} coordinates a Cys and His (or Asp) residue besides phenanthroline. In the same manner Cu^{2+}-phenanthroline may bind to the His and Glu residues in the galanin receptor. Since the Cu^{2+}-phenanthroline chelator complex had a higher binding affinity compared to free Cu^{2+}, additional binding energy appears to have been gained by an interaction between the phenanthroline moiety and the receptor.

Thus, as shown in Fig. 3, Cu^{2+}-ph nanthroline binds to the galanin receptor with an affinity (IC_{50} = 2 μM), which normally is found for lead compounds discovered through ordinary high throughput screening. It is therefore believed that Cu^{2+}-phenanthroline or related metal ion complexes may function as lead compounds in the search for high affinity galanin antagonists. Through ordinary mutational analysis of the potential metal-ion binding residues shown in Fig. 2, the binding site for the metal-ion chelator complex can then be mapped. Based on molecular models of the binding site for the metal-ion chelator complex, and guided by detailed knowledge of the structure of the main ligand-binding crevice in 7TM receptors as such obtained during multiple efforts in metal-site engineering, residues can be identified in the galanin receptor toward which chemical libraries of analogs of the chelator can be constructed. The wild-type galanin receptor can then be used to screen these libraries of metal-ion complexes. Further knowledge of the binding pocket in the galanin receptor may be obtained by determining residues involved in binding of other small metal ion chelates and by elucidation of the mechanism behind the copper binding.

Mapping of natural metal ion binding sites – During the last 10 years detailed knowledge has been obtained about mutational mapping of ligand-binding sites in 7TM receptors (Schwartz 1994, op.cit.). In the case of metal ion binding sites, this can be done simply by identifying the potential metal ion binding residues in the main ligand-binding crevice of the receptor and one by one substituting these by a residue with no or less ability to bind metal ions, either Ala or a more structurally similar residue, e.g. His to Gln, Cys to Ser, and Asp to Asn. This has previously been done in the mapping of the naturally occurring metal ion binding site at positions V:01 and V:05 in the NK3 receptor (Rosenkilde et al., *FEBS Lett.* 1998 Nov 13; 439(1-2), 35-40 98).

Screening of libraries of metal ion chelates in a metal ion binding site in a 7TM receptor – bipyridyl binds with an affinity of 10 μM to the [Asp113;CysVI:16;CysVII:06] site in the beta2-adrenergic receptor. As shown in Fig. 4, substitution of biquinoline with 2-phenyl-1,4-oxazoline or diethanolamide did neither impair nor improve the affinity to this metal ion binding site. However, substitution with benzamidine increased the affinity of the compound 20-fold. Thus, conceivably the 4-benzamidinyl substituted moiety has picked up additional chemical interaction sites in the receptor. Through classical mutational analysis of the surrounding residues it can be identified which points of interaction are responsible for this increase in binding affinity.

EXPERIMENTAL

Th human β_2-adr nergic receptor:

Materials and methods

Ligands. 1,10-phenanthroline and 2,2'-bipyridine were obtained from Sigma Chemical Co., St. Louis, MO. Zn^{2+}(phenanthroline)$_3$ and Cu^{2+}(phenanthroline)$_3$ were prepared by dissolving phenanthroline in ethanol and mixing with aqueous solutions of $ZnCl_2$ or $CuSO_4$ to a final molar ratio of 3:1.

Molecular Biology. The point mutations were constructed using oligonucleotide-directed mutagenesis and recombinant polymerase chain reaction. cDNAs encoding wild-type and mutant receptors were cloned into the eukaryotic expression vector pTEJ-8; all mutations were verified by restriction endonuclease mapping and DNA sequencing (ALFexpress DNA Sequencer, Amersham-Pharmacia biotech).

Cell biology. Cloned receptors were transiently expressed in COS-7 cells transfected 2 days before analysis. COS-7 cells were grown in Dulbecco's modified Eagle medium 1885 supplemented with 10 % fetal calf serum, 2 mM glutamine, and 10 :g/ml gentamicin. *Determination of intracellular cAMP accumulation.* COS-7 cells were seeded in six-well culture dishes one day after transfection at a density of 400.000 cells/well and supplemented with 2 :Ci [^{3}H]-adenine / ml (Amersham-Pharmacia biotech). Two days after transfection, cells were washed twice with HBS (25 mM HEPES, 0.75 mM NaH_2PO_4, 140 mM NaCl, pH 7.2) and incubated in HBS buffer supplemented with 1 mM 3-isobutyl-1-methylxanthine. Pindolol, catecholamine analogs, free metal ion or metal ion chelate was added and the cells were incubated for 30 min at 37 EC. The assay was terminated by aspirating the buffer followed by addition of ice-cold 5% trichloroacetic acid containing 0.1 mM unlabeled cAMP and ATP. cAMP was isolated by applying the supernatant to a 50W-X4 resin (Bio-Rad, Richmond, CA) followed by an alumina resin (A-9003, Sigma Chemical Co., St. Louis, MO). Determinations were made in duplicate. Since a small population of endogenous catecholamine receptors is present in COS-7 cells, which are stimulated by isoproterenol but not by pindolol, the latter was used as agonist in the present study.

Molecular Graphics. The coordinates for models of the prototypic 7TM receptor rhodopsin were generously provided by Drs. R.E. Hubbard and P. Herzyk (Herzyk, P. & Hubbard, R.E. (1998) *J.Mol.Biol.* **281**, 741-754.) and Dr. J.M. Baldwin (Baldwin, J.M., Schertler, G.F. & Unger, V.M. (1997) *J.Mol.Biol.* **272**, 144-164.). In both models Ala^{117} and Ala^{292} corresponding to Asp^{113} and Asn^{312} of the β_2-adrenergic receptor were substi-

tuted with a His and a Cys residue, respectively. Molecular graphics was done using W b-Lab Vi werPr , Molecular Simulations, Inc.

Data analysis. The cAMP curves were analysed and EC_{50} values determined by computerized non-linear regression using GraphPad Prizm (GraphPad Software, Inc., San Diego, CA).

The human galanin receptor 1:

Effect of Metal Ions on the Binding of Phenanthroline to the human Galanin Receptor 1

Transfections and Tissue Culture - COS-7 cells were grown at 10% CO_2 and 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. Transfection of the COS-7 cells was performed by the calcium phosphate precipitation method (Rosenkilde, MM. et al. (1994) *J. Biol. Chem.* **269**, 28160-28164).

Binding Experiments- COS-7 cells were transferred to culture plates 1 day after transfection. The number of cells seeded per well was determined by the apparent expression efficiency of the individual clones; the goal was to obtain 5-10% specific binding of the added radioactive ligand. Two days after transfection, cells were assayed by competition binding performed on whole cells for 3 h at 4 °C using ^{125}I-Galanin plus variable amounts of unlabeled ligand in 0.4 ml of 25 mM HEPES, 2.5 mM $MgCl_2$, 100 µg/ml bacitracin, pH 7.4, supplemented with 0.1% (w/v) bovine serum albumin. After incubation, cells were washed two times in 4 °C binding buffer without bacitracin. Non-specific binding was determined as the bindingin the presence of 0.1 µM unlabeled galanin. Determinations were made in duplicate. Every set of experiments was performed in parallel with a homologous competition binding curve where unlabeled galanin competed with iodinated galanin.

Calculations - IC_{50} values were determined by non-linear regression, and B_{max} values were calculated using Prism 3.0 (GraphPAD Software for Science, San Diego).

In heterologous binding experiments, Cu^{2+} and Zn^{2+} competed for [^{125}I]galanin binding having an IC_{50} value of 26 µM (Cu^{2+}) and 74 µm (Zn^{2+}), respectively. Both Cu^{2+} and Zn^{2+} exhibited a steep competition curve with a Hill coefficient of -3.6, an indication of complex/cooperative binding of these free metal ions.

Phenanthroline, which possess moderate affinity for the CXCR4 rec ptor (IC_{50}=50 μM), did not bind to th galanin receptor. However, in compl x with Cu^{2+} or Zn^{2+}, phenanthroline exhibited an affinity for the receptor that was higher compared to the free metal ions (IC_{50} = 2 μM for $Cu(Phe)_3$ and IC_{50} = 44 μM). In addition, the Hill-coefficient of the metal-chelator complex was reduced, indicating a 'one-site' competition binding when the metal was chelated by phenanthroline (Hill coefficient = -1.1).

CLAIMS

1. A method of identifying a membrane protein comprising a m tal ion binding site, the method comprising contacting a membrane protein with a test compound which comprises a moiety including at least one heteroatom for chelating a metal ion and further comprising a metal ion chelated thereto for binding to at least one amino acid residue of said membrane protein, under conditions permitting binding of the test compound to the membrane protein, and determining any binding of said test compound to said membrane protein.

2. A method according to claim 1, wherein the test compound is contacted with two or more membrane proteins for identification of possible metal ion binding sites thereof.

3. A method according to claim 1, wherein the membrane protein comprises at least one transmembrane domain.

4. A method according to claim 3, wherein the membrane protein comprises 1-14 transmembrane domains.

5. A method according to claim 4, wherein the membrane protein comprises one transmembrane domain, e.g. a growth factor receptor such as the growth hormone, insulin, epidermal growth factor, transforming growth factor, erythropoietin, granulocyte colony stimulating factor, TrkA and TrkB receptor.

6. A method according to claim 4, wherein the membrane protein comprises two transmembrane domains, e.g. a purinergic ion channel.

7. A method according to claim 4, wherein the membrane protein comprises 3, 4 or 5 transmembrane domains, e.g. a ligand-gated ion channel, such as nicotinic acetylcholine receptors, GABA receptors or glutamate receptors (NMDA or AMPA).

8. A method according to claim 4, wherein the membrane protein comprises 6 transmembrane domains, e.g. a voltage-gated ion channel, such as potassium, sodium, chloride or calcium channels.

9. A method according to claim 4, wherein the membrane protein comprises 7 transmembrane domains, e.g. a G-protein coupled receptor, such as acetylcholine, adenosine, adrenoceptors, anaphylatoxin chemotactic receptor, angiotensin, bombesin (neuromedin), bradykinin, calcitonin, calcitonin gene related peptide, conopressin, corticotropin releasing factor, amylin, adrenomedullin, calcium, cannabinoid, chemokine, cholecystokinin, conopressin, corticotropin-releasing factor, dopamine, eicosanoid, endothelin, fMLP, $GABA_B$, galanin, gastrin, gastric inhibitory peptide, glucagon, glucagon-like, glutamate, glycoprotein hormone (e.g. FSH, LSH, TSH, LH), gonadotropin releasing hormone, growth hormone releasing hormone, growth hormone secretagogue, histamine, 5-hydroxytryptamine, leukotriene, lysophospholipid, melanocortin, melatonin, neuropeptide Y, neurotensin, olfactory, opioid, opioid-like, opsin, orexin, oxytocin, parathyroid hormone/parathyroid hormone-related, P2Y, pheromone, platelet-activating factor, prostanoid, protease-activated, secretin, somatostatin, tachykinin, thrombin and protease activated, thyrotropin-releasing hormone, pituitary adenylate activating peptide, vasopressin, vasoactive intestinal peptide and viral encoded receptors; in particular galanin, P2Y, chemokine, melanocortin, bombesin, cannabinoid, lysophospholipid, fMLP, neuropeptide Y, tachykinin, dopamine, histamine, 5-hydroxytryptamine, histamine, mas-proto-oncogene, acetylcholine, oxytocin, herpes virus encoded, epstein-barr virus encoded, cytomegalovirus encoded and bradykinin receptors; preferably galanin receptor type 1, leukotriene B4 receptor, CXCR5, melanocortin-1 receptor, melanocortin-3 receptor, melanocortin-4 receptor, melanocortin-5 receptor, bombesin receptor subtype 3, cannabinoid receptor 1, cannabinoid receptor 2, EDG-2, EDG-4, FMLP-related receptor I, FMLP-related receptor-II, Y6 receptor, Y5 receptor, NK-1 receptor, NK-3 receptor, D2 receptor (short), D2 receptor (long), D4 receptor (D2C), duffy antigen, Q89609 herpes virus type 2, VU51_HSV6U, histamine H1 receptor, MAS proto-oncogene, muscarinic M1 receptor, muscarinic M2 receptor, muscarinic M3 receptor, muscarinic M5 receptor, oxytocin receptor, XCR1 receptor, EBI2 receptor, RDC1 receptor, O76000 receptor, O15218 receptor, GPR12 receptor or GPR3 receptor.

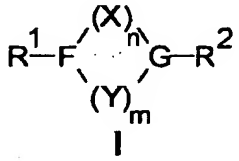
10. A method according to claim 4, wherein the membrane protein comprises 8, 10, 12 or 14 transmembrane domains, e.g. a transporter protein, such as GABA, monoamine or nucleoside transporter protein.

11. A method according to claim 1, wherein the membrane protein is a cell adhesion molecule, e.g. a NCAM, VCAM or ICAM.

12. A method according to claim 1, wherein the membrane protein is an enzyme such as adenylyl cyclase.

13. A method according to claim 1, wherein the test compound comprises a heteroalkyl or heterocyclyl moiety for chelating the metal ion.

14. A method according to claim 13, wherein the heteroalkyl or heterocyclyl moiety has the general formula I



I

wherein F is N, O or S and G is N, O or S;

R^1 is alkyl, aryl, cycloalkyl, alkoxy, heteroalkyl or heteroaryl, optionally substituted with one or more substituents selected from halogen, nitro, cyano, amino, alkyl, alkoxy, carboxy, amide or sulfonamide;

R^2 is alkyl, aryl, cycloalkyl, alkoxy, heteroalkyl or heteroaryl, optionally substituted with one or more substituents selected from halogen, nitro, cyano, amino, alkyl, alkoxy, carboxy, amide or sulfonamide;

R^1 and/or R^2 optionally forming a fused ring together with the heteroatom to which it is attached, or R^1 and/or R^2 optionally forming a fused ring together with the heteroatom to which it is attached and a carbon atom of X or Y;

X is $-CH_2-$, CH_2-CH_2-, $-CH_2-S-CH_2-$, $-CH_2-N-CH_2-$, $-CH=CH-CH=CH-$, $-CH_2-(Z)_a-(P)_c-(W)_b-CH_2-$, $-CH_2-O-CH_2-$, wherein

each of Z and W are independently C, S, O or N, and

P is -CH- or $-CH_2-$;

Y is $-CH_2-$, CH_2-CH_2-, $-CH_2-S-CH_2-$, $-CH_2-N-CH_2-$, $-CH=CH-CH=CH-$, $-CH_2-(Z)_a-(P)_c-(W)_b-CH_2-$, $-CH_2-O-CH_2-$, wherein

each of Z and W are independently C, S, O or N, and

P is -CH- or $-CH_2-$;

n is 0 or an integer of 1-5,

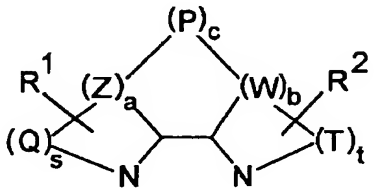
m is 0 or an integer of 1-5,

a is an integer of 1-3,

b is an integer of 1-3, and

c is an integer of 1-7.

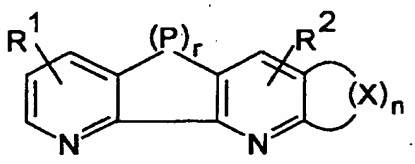
15. A method according to claim 14, wherein the test compound is on in which the heterocyclic moiety has the general formula III



III

wherein R^1, R^2, Z, W, P, a, b and c are as indicated above, and
each of Q and T is independently -CH- or $-CH_2-$, s is an integer of 1-7, and t is an integer of 1-7.

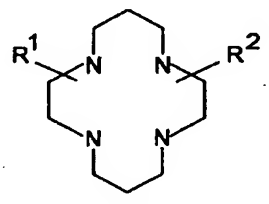
16. A method according to claim 14, wherein the test compound is one in which the heterocyclic moiety has the general formula IV



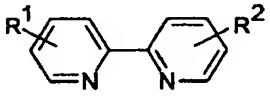
IV

wherein R^1, R^2, P, X and n are as indicated in claim 14, and r is an integer of 1-3.

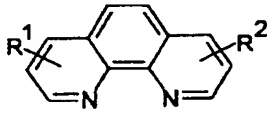
17. A method according to claim 14, wherein the test compound is one in which the heterocyclic moiety is selected from a compound of formula IIa, IIb or IIc



IIa



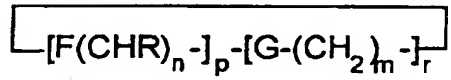
IIb



IIc

wherein R^1 and R^2 are as indicated in claim 14.

18. A method according to claim 14, wherein the test compound is one in which the heterocyclic moiety is selected from a compound of formula V



V

wherein F is N,O or S and G is N,O or S,
n is an integer from 1 to 5
m is 0 or an integer from 1 to 5,
p is 0 or an integer from 1 to 8,
r is 0 or an integer from 1 to 8, and
R is alkyl, aryl, cycloalkyl, alkoxy, heteroalkyl or heteroaryl, optionally substituted with one or more substituents selected from halogen, nitro, cyano, amino, alkyl, alkoxy, carboxy, amide or sulfonamide.

19. A method according to claim 1, wherein the metal ion is one that binds to an amino acid residue containing an electron donating atom such as S, O and N, or with an aromatic amino acid residue.

20. A method according to claim 19, wherein the amino acid residue is selected from the group consisting of Ser, Lys, Arg, Tyr, Thr, Trp, Phe, Asp, Glu, Asn, Gln, Cys and His, in particular Asp, Glu, Cys and His, preferably His.

21. A method according to claim 19, wherein the metal ion is selected from the group consisting of rubidium, caesium, beryllium, magnesium, calcium, strontium, barium, scandium, yttrium, lutetium, titanium, zirconium, hafnium, vanadium, niobium, tantalum, chromium, molybdenum, tungsten, manganese, technetium, rhenium, iron, ruthenium, osmium, cobalt, rhodium, iridium, nickel, palladium, platinum, copper, silver, gold, zinc, cadmium, mercury, boron, aluminium, gallium, indium, thallium, silicon, germanium, tin, lead, arsenic, antimony, bismuth, tellurium, polonium, astatine, lanthanum, cerium, praseodymium, neodymium, promethium, samarium, europium, gadolinium, terbium, dyspro-

sium, holmium, erbium, thulium, ytterbium, thorium and oxidation states or isotopes thereof; in particular rubidium, magnesium, calcium, strontium, barium, yttrium, lutetium, chromium, manganese, technetium, rhenium, iron, ruthenium, osmium, cobalt, rhodium, nickel, palladium, platinum, copper, silver, gold, zinc, aluminium, gallium, indium, thallium, germanium, tin, antimony, bismuth samarium, europium, gadolinium, terbium, thorium and oxidation states and isotopes thereof; preferably magnesium (II), calcium (II), manganese (II), iron (II) and (III), ruthenium (II) and (III), nickel (II), palladium (II), platinum (II), copper (II), zinc (II), samarium (III), europium (III), terbium (III) or isotopes thereof.

22. A method according to claim 21, wherein the heterocyclic test compound is a Cu^{2+}-phenanthroline complex, a Zn^{2+}-phenanthroline complex, a Mg^{2+}-phenanthroline complex, a Ca^{2+}-phenanthroline complex, a Cu^{2+}-bipyridyl complex, a Zn^{2+}-bipyridyl complex, a Mg^{2+}-bipyridyl complex, a Ca^{2+}-bipyridyl complex, a Cu^{2+}-1,4,8,11-tetraazacyclotetradecane, a Zn^{2+}-1,4,8,11-tetraazacyclotetradecane, a Mg^{2+}-1,4,8,11-tetraazacyclotetradecane, a Ca^{2+}-1,4,8,11-tetraazacyclotetradecane.bipyridylbipyridyl

23. A method of identifying a membrane protein comprising a metal ion binding site, the method comprising

(a) selecting a nucleotide sequence suspected of coding for a membrane protein and deducing the amino acid sequence thereof,

(b) expressing said nucleotide sequence in a suitable host cell,

(c) contacting said cell or a portion thereof including the expressed membrane protein with a test compound which comprises a moiety including at least one heteroatom for chelating a metal ion and further comprising a metal ion chelated thereto for binding to at least one amino acid residue of said membrane protein, under conditions permitting binding of the test compound to the membrane protein, and determining any binding of said test compound to said membrane protein, and

(d) determining, based on the generic three-dimensional model of the class of membrane proteins to which the membrane protein or suspected membrane protein belongs, at least one metal ion binding amino acid residue located in said membrane protein to locate the metal ion binding site of said membrane protein.

24. A method of mapping a metal ion binding site of a membrane protein, the method comprising

(a) contacting membrane protein with a test compound which comprises a moiety including at least one heteroatom for chelating a metal ion and further comprising a metal ion chelated thereto for binding to at least one amino acid residue of said membrane protein, under conditions permitting binding of the test compound to the membrane protein, and determining any binding of said test compound to said membrane protein, and
(b) determining, based on the primary structure of the specific membrane protein in question and the generic three-dimensional model of the class of membrane proteins to which the specific membrane protein of step (a) belongs, at least one metal ion binding amino acid residue located in the membrane protein to identify the metal ion binding site of said membrane protein.

25. A method according to claim 24, wherein the determination of step (a) is performed using competition with binding of a labelled ligand of the membrane protein, detection of any changes in signal transduction from the membrane protein, or using a chelating agent which is in itself detectable or labelled with a detectable labelling agent.

26. A method according to claim 24, wherein the determination of step (b) is performed using a three-dimensional model of the membrane protein showing the spatial arrangement of the amino acid residues defining the area of the metal ion binding site, site-directed mutagenesis of a least one amino acid residue potentially involved in interaction with said metal ion chelated to the test compound, followed by expression of the mutated membrane protein in a suitable cell, contacting said cell or a portion thereof including the expressed mutated membrane protein with the test compound, and determining any effect on binding in a competitive binding assay using a labelled ligand of the membrane protein, detection of any changes in signal transduction from the membrane protein or using a chelating agent which is in itself detectable or labelled with a detectable labelling agent.

27. A method according to claim 24 comprising a further step of determining at least one other amino acid residue of the membrane protein binding at least one functional group of the test compound other than the metal ion.

28. A method according to claim 27, wherein the determination is performed using site-directed mutagenesis of a least one amino acid residue potentially involved in interaction with said functional group of the test compound other than the metal ion, followed by expression of the mutated membrane protein in a suitable cell, contacting said cell or a por-

tion thereof including the mutated m mbrane protein with the test compound, and determining any effect on binding in a competitive binding assay using a labelled ligand of the membran protein, detection of any changes in signal transduction from the membrane protein or using a chelating agent which is in itself detectable or labelled with a detectable labelling agent.

29. A method according to claim 24, wherein the membrane protein comprises at least one transmembrane domain.

30. A method according to claim 29, wherein the membrane protein comprises 1-14 transmembrane domains.

31. A method according to claim 30, wherein the membrane protein comprises one transmembrane domain, e.g. a growth factor receptor such as the growth hormone, insulin, epidermal growth factor, transforming growth factor, erythropoietin, granulocyte colony stimulating factor, TrkA and TrkB receptor.

32. A method according to claim 30, wherein the membrane protein comprises two transmembrane domains, e.g. a purinergic ion channel.

33. A method according to claim 30, wherein the membrane protein comprises 3, 4 or 5 transmembrane domains, e.g. a ligand-gated ion channel, such as nicotinic acetylcholine receptors, GABA receptors or glutamate receptors (NMDA or AMPA).

34. A method according to claim 30, wherein the membrane protein comprises 6 transmembrane domains, e.g. a voltage-gated ion channel, such as potassium, sodium, chloride or calcium channels.

35. A method according to claim 30, wherein the membrane protein comprises 7 transmembrane domains, e.g. a G-protein coupled receptor, such as acetylcholine, adenosine, adrenoceptors, anaphylatoxin chemotactic receptor, angiotensin, bombesin (neuromedin), bradykinin, calcitonin, calcitonin gene related peptide, conopressin, corticotropin releasing factor, amylin, adrenomedullin, calcium, cannabinoid, chemokine, cholecystokinin, conopressin, corticotropin-releasing factor, dopamine, eicosanoid, endothelin, fMLP, $GABA_B$, galanin, gastrin, gastric inhibitory peptide, glucagon, glucagon-like, glutamate, glycopro-

tein hormone (e.g. FSH, LSH, TSH, LH), gonadotropin releasing hormone, growth hormone releasing hormone, growth hormone s cretagogu , histamine, 5-hydroxytryptamine, leukotriene, lysophospholipid, m lanocortin, melatonin, neuropeptide Y, neurotensin, olfactory, opioid, opioid-like, opsin, orexin, oxytocin, parathyroid hormone/parathyroid hormone-related, P2Y, pheromone, platelet-activating factor, prostanoid, protease-activated, secretin, somatostatin, tachykinin, thrombin and protease activated, thyrotropin-releasing hormone, pituitary adenylate activating peptide, vasopressin, vasoactive intestinal peptide and viral encoded receptors; in particular galanin, P2Y, chemokine, melanocortin, bombesin, cannabinoid, lysophospholipid, fMLP, neuropeptide Y, tachykinin, dopamine, histamine, 5-hydroxytryptamine, histamine, mas-proto-oncogene, acetylcholine, oxytocin, herpes virus encoded, epstein-barr virus encoded, cytomegalovirus encoded and bradykinin receptors; preferably galanin receptor type 1, leukotriene B4 receptor, CXCR5, melanocortin-1 receptor, melanocortin-3 receptor, melanocortin-4 receptor, melanocortin-5 receptor, bombesin receptor subtype 3, cannabinoid receptor 1, cannabinoid receptor 2, EDG-2, EDG-4, FMLP-related receptor I, FMLP-related receptor-II, Y6 receptor, Y5 receptor, NK-1 receptor, NK-3 receptor, D2 receptor (short), D2 receptor (long), D4 receptor (D2C), duffy antigen, Q89609 herpes virus type 2, VU51_HSV6U, histamine H1 receptor, MAS proto-oncogene, muscarinic M1 receptor, muscarinic M2 receptor, muscarinic M3 receptor, muscarinic M5 receptor, oxytocin receptor, XCR1 receptor, EBI2 receptor, RDC1 receptor, O76000 receptor, O15218 receptor, GPR12 receptor or GPR3 receptor.

36. A method according to claim 30, wherein the membrane protein comprises 8, 10, 12 or 14 transmembrane domains, e.g. a transporter protein, such as GABA, monoamine and nucleoside transporter proteins.

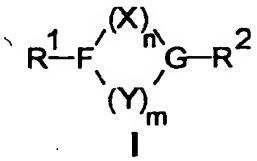
37. A method according to claim 24, wherein the membrane protein is a cell adhesion molecule, e.g. a NCAM, VCAM or ICAM.

38. A method according to claim 24, wherein the membrane protein is a multidrug resistance protein, e.g. a P-glycoprotein, multidrug resistance associated protein, lung resistance related protein, drug resistance associated protein, breast cancer resistance protein, adenosine triphosphate-binding cassette protein, Bmr, QacA or EmrAB/TolC.

39. A method according to claim 1, wherein the membrane protein is an enzyme such as adenylyl cyclase.

40. A method according to claim 24, wherein the test compound comprises a heteroalkyl or heterocyclyl moiety for chelating the metal ion.

41. A method according to claim 40, wherein the heteroalkyl or heterocyclyl moiety has the general formula I



I

wherein F is N, O or S and G is N, O or S;

R^1 is alkyl, aryl, cycloalkyl, alkoxy, heteroalkyl, heterocycloalkyl or heteroaryl, optionally substituted with one or more substituents selected from halogen, nitro, cyano, amino, alkyl, alkoxy, carboxy, amide or sulfonamide;

R^2 is alkyl, aryl, cycloalkyl, alkoxy, heteroalkyl or heteroaryl, optionally substituted with one or more substituents selected from halogen, nitro, cyano, amino, alkyl, alkoxy, carboxy, amide or sulfonamide;

R^1 and/or R^2 optionally forming a fused ring together with the heteroatom to which it is attached, or R^1 and/or R^2 optionally forming a fused ring together with the heteroatom to which it is attached and a carbon atom of X or Y;

X is $-CH_2-$, CH_2-CH_2-, $-CH_2-S-CH_2-$, $-CH_2-N-CH_2-$, $-CH=CH-CH=CH-$, $-CH_2-(Z)_a-(P)_c-(W)_b-CH_2-$, $-CH_2-O-CH_2-$, wherein

each of Z and W are independently C, S, O or N, and

P is -CH- or $-CH_2-$;

Y is $-CH_2-$, CH_2-CH_2-, $-CH_2-S-CH_2-$, $-CH_2-N-CH_2-$, $-CH=CH-CH=CH-$, $-CH_2-(Z)_a-(P)_c-(W)_b-CH_2-$, $-CH_2-O-CH_2-$, wherein

each of Z and W are independently C, S, O or N, and

P is -CH- or $-CH_2-$;

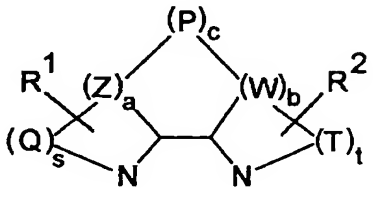
n is 0 or an integer of 1-5,

m is 0 or an integer of 1-5,

a is an integer of 1-3,

b is an integer of 1-3, and
c is an integer of 1-7.

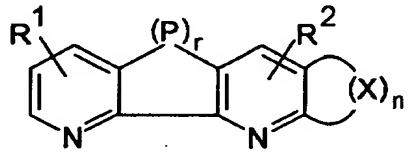
42. A method according to claim 41, wherein the test compound is one in which the heterocyclic moiety has the general formula III



III

wherein R^1, R^2, Z, W, P, a, b and c are as indicated above, and
each of Q and T is independently -CH- or $-CH_2-$, s is an integer of 1-7, and t is an integer of 1-7.

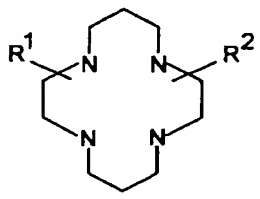
43. A method according to claim 42, wherein the test compound is one in which the heterocyclic moiety has the general formula IV



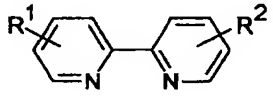
IV

wherein R^1, R^2, P, X, T and n are as indicated in claim 37, and r is an integer of 1-3.

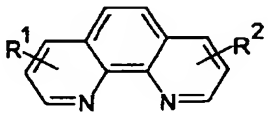
44. A method according to claim 41, wherein the test compound is one in which the heterocyclic moiety is selected from a compound of formula IIa, IIb or IIc



IIa



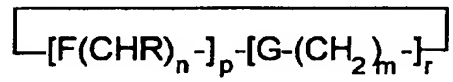
IIb



IIc

wherein R^1 and R^2 are as indicated in claim 41.

45. A method according to claim 41, wherein the test compound is one in which the heterocyclic moiety is selected from a compound of formula V:



V

wherein F is N, O or S and
G is N, O or S,
n is 0 or an integer from 1 to 5,
m is 0 or an integer from 1 to 5,
p is 0 or an integer from 1 to 8,
r is 0 or an integer from 1 to 8, and
R is alkyl, aryl, cycloalkyl, alkoxy, heteroalkyl or heteroaryl, optionally substituted with one or more substituents selected from halogen, nitro, cyano, amino, alkyl, alkoxy, carboxy, amide or sulfonamide.

46. A method according to claim 41, wherein the test compound is one in which the heterocyclic moiety is selected from a compound shown in Table 1.

47. A method according to claim 24, wherein the metal ion is one that binds to an amino acid residue containing an electron donating atom such as S, O and N, or an aromatic amino acid residue.

48. A method according to claim 47, wherein the amino acid residue capable of binding the metal ion is selected from the group consisting of Ser, Lys, Arg, Tyr, Thr, Trp, Phe, Asp, Glu, Asn, Gln, Cys and His, in particular Asp, Glu, Cys and His, preferably His.

49. A method according to claim 47, wherein the metal ion is selected from the group consisting of rubidium, caesium, beryllium, magnesium, calcium, strontium, barium, scandium, yttrium, lutetium, titanium, zirconium, hafnium, vanadium, niobium, tantalum, chromium, molybdenum, tungsten, manganese, technetium, rhenium, iron, ruthenium,

osmium, cobalt, rhodium, iridium, nickel, palladium, platinum, copper, silver, gold, zinc, cadmium, mercury, boron, aluminium, gallium, indium, thallium, silicon, germanium, tin, lead, arsenic, antimony, bismuth, tellurium, polonium, astatine, lanthanum, cerium, praseodymium, neodymium, promethium, samarium, europium, gadolinium, terbium, dysprosium, holmium, erbium, thulium, ytterbium, thorium and oxidation states or isotopes thereof; in particular rubidium, magnesium, calcium, strontium, barium, yttrium, lutetium, chromium, manganese, technetium, rhenium, iron, ruthenium, osmium, cobalt, rhodium, nickel, palladium, platinum, copper, silver, gold, zinc, aluminium, gallium, indium, thallium, germanium, tin, antimony, bismuth samarium, europium, gadolinium, terbium, thorium and oxidation states or isotopes thereof; preferably magnesium (II), calcium (II), manganese (II), iron (II) and (III), ruthenium (II) and (III), nickel (II), palladium (II), platinum (II), copper (II), zinc (II), samarium (III), europium (III), terbium (III) or isotopes thereof.

50. A method according to claim 49, wherein the heterocyclic test compound is a Cu^{2+}-phenanthroline complex, a Zn^{2+}-phenanthroline complex, a Mg^{2+}-phenanthroline complex, a Ca^{2+}-phenanthroline complex, a Cu^{2+}-bipyridyl complex, a Zn^{2+}-bipyridyl complex, a Mg^{2+}-bipyridyl complex, a Ca^{2+}-bipyridyl complex, a Cu^{2+}-1,4,8,11-tetraazacyclotetradecane, a Zn^{2+}-1,4,8,11-tetraazacyclotetradecane, a Mg^{2+}-1,4,8,11-tetraazacyclotetradecane, a Ca^{2+}-1,4,8,11-tetraazacyclotetradecane.

51. A method according to any of claims 24-50, wherein the membrane protein is a receptor, in particular a 7 transmembrane domain (7TM) receptor.

52. A method according to claim 51, wherein the 7TM receptor is a acetylcholine, adenosine, adrenoceptors, anaphylatoxin chemotactic receptor, angiotensin, bombesin (neuromedin), bradykinin, calcitonin, calcitonin gene related peptide, conopressin, corticotropin releasing factor, amylin, adrenomedullin, calcium, cannabinoid, chemokine, cholecystokinin, conopressin, corticotropin-releasing factor, dopamine, eicosanoid, endothelin, fMLP, $GABA_B$, galanin, gastrin, gastric inhibitory peptide, glucagon, glucagon-like, glutamate, glycoprotein hormone (e.g. FSH, LSH, TSH, LH), gonadotropin releasing hormone, growth hormone releasing hormone, growth hormone secretagogue, histamine, 5-hydroxytryptamine, leukotriene, lysophospholipid, melanocortin, melatonin, neuropeptide Y, neurotensin, olfactory, opioid, opioid-like, opsin, orexin, oxytocin, parathyroid hormone/parathyroid hormone-related, P2Y, pheromone, platelet-activating factor, prostanoid, protease-activated, secretin, somatostatin, tachykinin, thrombin and protease activated,

thyrotropin-releasing hormone, pituitary adenylate activating peptide, vasopressin, vasoactive intestinal peptide and viral encoded receptors; in particular galanin, P2Y, chemokine, melanocortin, bombesin, cannabinoid, lysophospholipid, fMLP, neuropeptide Y, tachykinin, dopamine, histamine, 5-hydroxytryptamine, histamine, mas-proto-oncogene, acetylcholine, oxytocin, herpes virus encoded, epstein-barr virus encoded, cytomegalovirus encoded and bradykinin receptors; preferably galanin receptor type 1, leukotriene B4 receptor, CXCR5, melanocortin-1 receptor, melanocortin-3 receptor, melanocortin-4 receptor, melanocortin-5 receptor, bombesin receptor subtype 3, cannabinoid receptor 1, cannabinoid receptor 2, EDG-2, EDG-4, FMLP-related receptor I, FMLP-related receptor-II, Y6 receptor, Y5 receptor, NK-1 receptor, NK-3 receptor, D2 receptor (short), D2 receptor (long), D4 receptor (D2C), duffy antigen, Q89609 herpes virus type 2, VU51_HSV6U, histamine H1 receptor, MAS proto-oncogene, muscarinic M1 receptor, muscarinic M2 receptor, muscarinic M3 receptor, muscarinic M5 receptor, oxytocin receptor, XCR1 receptor, EBI2 receptor, RDC1 receptor, O76000 receptor, O15218 receptor, GPR12 receptor or GPR3 receptor.

53. A method according to claim 52, wherein the receptor is galanin receptor 1.

54. A method according to claim 53, wherein the receptor is human galanin receptor 1.

55. A method according to claim 54, wherein His 267 and Glu271 of human galanin receptor 1 when subjected individually to site-directed mutagenesis and tested for binding are involved in metal ion binding, determined as loss of binding when contacted with Cu^{2+}-phenanthroline complex or Zn^{2+}-phenathroline complex.

56. A method according to claim 55, wherein Phe115 of human galanin receptor 1 when subjected to site-directed mutagenesis and tested for binding is involved in phenanthrolin binding, determined as loss of binding when contacted with Cu^{2+}-phenanthroline complex or Zn^{2+}-phenanthroline complex.

57. A method of screening for compounds capable of binding to a metal ion binding site of a membrane protein, the method comprising contacting a membrane protein with test compounds which comprise a moiety including at least one heteroatom for chelating a metal ion and further comprising a metal ion chelated thereto for binding to at least one other amino acid residue of said membrane protein, under conditions permitting binding of

the test compound to the membrane protein, and determining any binding of said test compound to said membrane protein.

58. A method according to claim 57 comprising the further step of determining, based on the primary structure of the specific membrane protein in question and the generic three-dimensional model of the class of membrane proteins to which the specific membrane protein belongs, at least one metal ion binding amino acid residue located in the membrane protein to identify the metal ion binding site of said membrane protein.

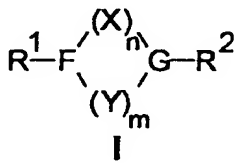
59. A method according to claim 58, wherein the determination is performed using a three-dimensional model of the membrane protein showing the spatial arrangement of the amino acid residues defining the area of the metal ion binding site, site-directed mutagenesis of a least one amino acid residue potentially involved in interaction with said metal ion chelated to the test compound, followed by expression of the mutated membrane protein in a suitable cell, contacting said cell or a portion thereof including the expressed mutated membrane protein with the test compound, and determining any effect on binding in a competitive binding assay using a labelled ligand of the membrane protein, detection of any changes in signal transduction from the membrane protein or using a chelating agent which is in itself detectable or labelled with a detectable labelling agent.

60. A method according to claim 57 or 58 comprising a further step of determining at least one other amino acid residue of the membrane protein binding at least one functional group of the test compound other than the metal ion.

61. A method according to claim 60, wherein the determination is performed using site-directed mutagenesis of a least one amino acid residue potentially involved in interaction with said functional group of the test compound other than the metal ion, followed by expression of the mutated membrane protein in a suitable cell, contacting said cell or a portion thereof including the mutated membrane protein with the test compound, and determining any effect on binding in a competitive binding assay using a labelled ligand of the membrane protein, detection of any changes in signal transduction from the membrane protein or using a chelating agent which is in itself detectable or labelled with a detectable labelling agent.

62. A method according to any of claims 57-61, wherein th test compounds comprise a h teroalkyl or heterocyclyl moiety for ch lating the metal ion.

63. A method according to claim 62, wherein the heteroalkyl or heterocyclyl moiety has the general formula I



I

wherein F is N, O or S and G is N, O or S;

R^1 is alkyl, aryl, cycloalkyl, alkoxy, heteroalkyl, heterocycloalkyl or heteroaryl, optionally substituted with one or more substituents selected from halogen, nitro, cyano, amino, alkyl, alkoxy, carboxy, amide or sulfonamide;

R^2 is alkyl, aryl, cycloalkyl, alkoxy, heteroalkyl or heteroaryl, optionally substituted with one or more substituents selected from halogen, nitro, cyano, amino, alkyl, alkoxy, carboxy, amide or sulfonamide;

R^1 and/or R^2 optionally forming a fused ring together with the nitrogen to which it is attached, or R^1 and/or R^2 optionally forming a fused ring together with the heteroatom to which it is attached and a carbon atom of X or Y;

X is $-CH_2-$, CH_2-CH_2-, $-CH_2-S-CH_2-$, $-CH_2-N-CH_2-$, $-CH=CH-CH=CH-$, $-CH_2-(Z)_a-(P)_c-(W)_b-CH_2-$, $-CH_2-O-CH_2-$, wherein

each of Z and W are independently C, S, O or N, and

P is -CH- or $-CH_2-$;

Y is $-CH_2-$, CH_2-CH_2-, $-CH_2-S-CH_2-$, $-CH_2-N-CH_2-$, $-CH=CH-CH=CH-$, $-CH_2-(Z)_a-(P)_c-(W)_b-CH_2-$, $-CH_2-O-CH_2-$, wherein

each of Z and W are independently C, S, O or N, and

P is -CH- or $-CH_2-$;

n is 0 or an integer of 1-5,

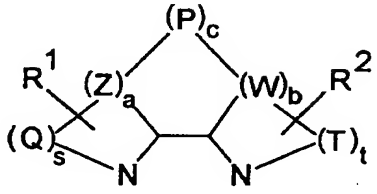
m is 0 or an integer of 1-5,

a is an integer of 1-3,

b is an integer of 1-3, and

c is an integer of 1-7.

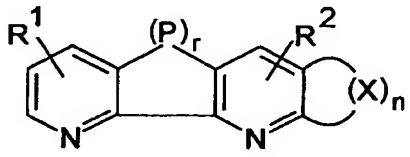
64. A method according to claim 63, wherein the t st compound is one in which the heterocyclic moiety has the general formula III



III

wherein R^1, R^2, Z, W, P, a, b and c are as indicated in claim 57, and each of Q and T is independently -CH- or -CH_2-, s is an integer of 1-7, and t is an integer of 1-7.

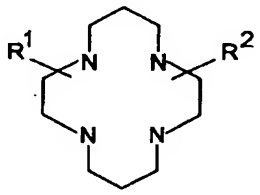
65. A method according to claim 64, wherein the test compound is one in which the heterocyclic moiety has the general formula IV



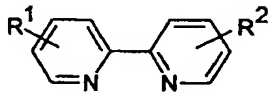
IV

wherein R^1, R^2, P, X and n are as indicated in claim 57, and r is an integer of 1-3.

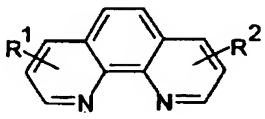
66. A method according to claim 63, wherein the test compound is one in which the heterocyclic moiety is selected from a compound of formula IIa, IIb or IIc



IIa



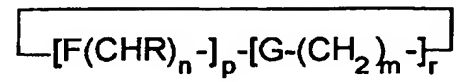
IIb



IIc

wherein R^1 and R^2 are as indicated in claim 59.

67. A method according to claim 63, wh rein the test compound is one in which the heterocyclic moiety is selected from a compound of formula V:



V

wherein F is N, O or S and
G is N, O or S,
n is 0 or an integer from 1 to 5,
m is 0 or an integer from 1 to 5,
p is 0 or an integer from 1 to 8,
r is 0 or an integer from 1 to 8, and
R is alkyl, aryl, cycloalkyl, alkoxy, heteroalkyl or heteroaryl, optionally substituted with one or more substituents selected from halogen, nitro, cyano, amino, alkyl, alkoxy, carboxy, amide or sulfonamide.

68. A method according to claim 63, wherein the heterocyclic moiety is selected from a compound shown in Table 1.

69. A method according to claim 57, wherein the metal ion is one reacting with an amino acid residue containing an electron donating atom such as S, O and N, or an aromatic amino acid residue.

70. A method according to claim 69, wherein the amino acid residue is selected from the group consisting of Ser, Lys, Arg, Tyr, Thr, Phe, Trp, Asp, Glu, Asn, Gln, Cys and His, in particular Asp, Glu, Cys and His, preferably His.

71. A method according to claim 69, wherein the metal ion is selected from the group consisting of rubidium, caesium, beryllium, magnesium, calcium, strontium, barium, scandium, yttrium, lutetium, titanium, zirconium, hafnium, vanadium, niobium, tantalum, chromium, molybdenum, tungsten, manganese, technetium, rhenium, iron, ruthenium, osmium, cobalt, rhodium, iridium, nickel, palladium, platinum, copper, silver, gold, zinc,

cadmium, mercury, boron, aluminium, gallium, indium, thallium, silicon, germanium, tin, lead, arsenic, antimony, bismuth, tellurium, polonium, astatine, lanthanum, cerium, praseodymium, neodymium, promethium, samarium, europium, gadolinium, terbium, dysprosium, holmium, erbium, thulium, ytterbium, thorium and oxidation states or isotopes thereof; in particular rubidium, magnesium, calcium, strontium, barium, yttrium, lutetium, chromium, manganese, technetium, rhenium, iron, ruthenium, osmium, cobalt, rhodium, nickel, palladium, platinum, copper, silver, gold, zinc, aluminium, gallium, indium, thallium, germanium, tin, antimony, bismuth samarium, europium, gadolinium, terbium, thorium and oxidation states or isotopes thereof; preferably magnesium (II), calcium (II), manganese (II), iron (II) and (III), ruthenium (II) and (III), nickel (II), palladium (II), platinum (II), copper (II), zinc (II), samarium (III), europium (III), terbium (III) or isotopes thereof.

72. A method according to claim 71, wherein the heterocyclic test compound is a Cu^{2+}-phenanthroline complex, a Zn^{2+}-phenanthroline complex, a Mg^{2+}-phenanthroline complex, a Ca^{2+}-phenanthroline complex, a Cu^{2+}-bipyridyl complex, a Zn^{2+}-bipyridyl complex, a Mg^{2+}-bipyridyl complex, a Ca^{2+}-bipyridyl complex, a Cu^{2+}-1,4,8,11-tetraazacyclotetradecane, a Zn^{2+}-1,4,8,11-tetraazacyclotetradecane, a Mg^{2+}-1,4,8,11-tetraazacyclotetradecane, a Ca^{2+}-1,4,8,11-tetraazacyclotetradecane.

73. A method according to claim 57, wherein the test compound is an agonist, partial agonist or positive modulator of the membrane protein.

74. A method according to claim 57, wherein the test compound is an antagonist, partial agonist, inverse agonist or negative modulator of the membrane protein.

75. A method according to any of claims 57-74, wherein the membrane protein comprises at least one transmembrane domain.

76. A method according to claim 75, wherein the membrane protein comprises 1-14 transmembrane domains.

77. A method according to claim 76, wherein the membrane protein comprises one transmembrane domain, e.g. a growth factor receptor such as the growth hormone, insulin, epidermal growth factor, transforming growth factor, erythropoietin, granulocyte colony stimulating factor, TrkA and TrkB receptor.

78. A method according to claim 76, wherein the membrane protein comprises two transmembrane domains, e.g. a purinergic ion channel.

79. A method according to claim 76, wherein the membrane protein comprises 3, 4 or 5 transmembrane domains, e.g. a ligand-gated ion channel, such as nicotinic acetylcholine receptors, GABA receptors or glutamate receptors (NMDA or AMPA).

80. A method according to claim 76, wherein the membrane protein comprises 6 transmembrane domains, e.g. a voltage-gated ion channel, such as potassium, sodium, chloride or calcium channels.

81. A method according to claim 76, wherein the membrane protein comprises 7 transmembrane domains, e.g. a G-protein coupled receptor, such as acetylcholine, adenosine, adrenoceptors, anaphylatoxin chemotactic receptor, angiotensin, bombesin (neuromedin), bradykinin, calcitonin, calcitonin gene related peptide, conopressin, corticotropin releasing factor, amylin, adrenomedullin, calcium, cannabinoid, chemokine, cholecystokinin, conopressin, corticotropin-releasing factor, dopamine, eicosanoid, endothelin, fMLP, $GABA_B$, galanin, gastrin, gastric inhibitory peptide, glucagon, glucagon-like, glutamate, glycoprotein hormone (e.g. FSH, LSH, TSH, LH), gonadotropin releasing hormone, growth hormone releasing hormone, growth hormone secretagogue, histamine, 5-hydroxytryptamine, leukotriene, lysophospholipid, melanocortin, melatonin, neuropeptide Y, neurotensin, olfactory, opioid, opioid-like, opsin, orexin, oxytocin, parathyroid hormone/parathyroid hormone-related, P2Y, pheromone, platelet-activating factor, prostanoid, protease-activated, secretin, somatostatin, tachykinin, thrombin and protease activated, thyrotropin-releasing hormone, pituitary adenylate activating peptide, vasopressin, vasoactive intestinal peptide and viral encoded receptors; in particular galanin, P2Y, chemokine, melanocortin, bombesin, cannabinoid, lysophospholipid, fMLP, neuropeptide Y, tachykinin, dopamine, histamine, 5-hydroxytryptamine, histamine, mas-proto-oncogene, acetylcholine, oxytocin, herpes virus encoded, epstein-barr virus encoded, cytomegalovirus encoded and bradykinin receptors; preferably galanin receptor type 1, leukotriene B4 receptor, CXCR5, melanocortin-1 receptor, melanocortin-3 receptor, melanocortin-4 receptor, melanocortin-5 receptor, bombesin receptor subtype 3, cannabinoid receptor 1, cannabinoid receptor 2, EDG-2, EDG-4, FMLP-related receptor I, FMLP-related receptor-II, Y6 receptor, Y5 receptor, NK-1 receptor, NK-3 receptor, D2 receptor (short), D2 receptor

(long), D4 receptor (D2C), duffy antig n, Q89609 h rpes virus type 2, VU51_HSV6U, histamine H1 receptor, MAS proto-oncogene, muscarinic M1 receptor, muscarinic M2 receptor, muscarinic M3 receptor, muscarinic M5 receptor, oxytocin receptor, XCR1 receptor, EBI2 receptor, RDC1 receptor, O76000 receptor, O15218 receptor, GPR12 receptor or GPR3 receptor.

82. A method according to claim 76, wherein the membrane protein comprises 8, 10, 12 or 14 transmembrane domains, e.g. a transporter protein, such as GABA, monoamine or nucleoside transporter proteins.

83. A method according to claim 57, wherein the membrane protein is a multidrug resistance pump, e.g. a Bmr, QacA or EmrAB/TolC.

84. A method according to claim 57, wherein the membrane protein is a cell adhesion molecule, e.g. a NCAM, VCAM or ICAM.

85. A method according to claim 57, wherein the membrane protein is an enzyme such as adenylyl cyclase.

86. A method according to claim 81, wherein the receptor is a galanin receptor.

87. A method according to claim 86, wherein the receptor is galanin receptor 1.

88. A method according to claim 87, wherein the receptor is human galanin receptor 1.

89. A method according to claim 88, wherein said test compound interacts with His267 and Glu271 of human galanin receptor 1.

90. A method according to claim 88, wherein said test compound interacts with Phe115 of human galanin receptor 1.

91. A method of preferentially stabilising a membrane protein comprising a metal ion binding site in an active conformation, the method comprising contacting said membrane protein with an effective amount of a heteroalkyl or heterocyclyl metal ion chelate acting as an agonist, partial agonist or positive modulator of the membrane protein.

92. A method of preferentially stabilising a membrane protein comprising a metal ion binding site in an inactive conformation, the method comprising contacting said membrane protein with an effective amount of a heteroalkyl or heterocyclyl metal ion chelate acting as an antagonist, partial antagonist, inverse agonist or negative modulator of the membrane protein.

93. A method of improving the binding affinity of a heteroalkyl or heterocyclyl metal ion chelate to a metal ion binding membrane protein, the method comprising
(a) selecting a heteroalkyl or heterocyclyl metal ion chelate with a binding affinity to a given membrane protein of 50 μM or better as identified by the screening method of claim 57,
(b) mapping the site of the membrane protein to which the chelate binds using the method of claim 24,
(c) determining at least one amino acid residue (potentially) involved in interaction with at least one functional group of the chelate,
(d) providing the chelate with one or more functional groups of a nature and in a position optimised for interaction with said amino acid residue to generate a library of chelate derivatives,
(e) screening the derivatives of step (d) by the method of claim 57, and
(f) optionally repeating any one or a combination of two or more of steps (a)-(e) one or more times to generate heteroalkyl or heterocyclyl metal ion chelating compound library with an improved binding affinity for the membrane protein.

94. A method according to claim 90, wherein the compound of step (e) or (f) has a binding affinity better than 10 μM, preferably better than 1 μM, more preferably better than 500 nM, in particular better than 100 nM.

95. A method of designing a compound library capable of binding to the metal ion binding site of a given membrane protein, the method comprising
(a) selecting a heteroalkyl or heterocyclyl metal ion chelate with a binding affinity to a given membrane protein of 50 μM or better as identified by the screening method of claim 57,
(b) mapping the site of the membrane protein to which the chelate binds using the method of claim 24,

(c) determining at least one amino acid residue (potentially) involved in interacti n with at least one functional group of the chelate,

(d) providing the chelate with one or more functional groups of a nature and in a position optimised for interaction with said amino acid residue to generate a library of chelate derivatives,

(e) screening the derivatives of step (d) by the method of claim 57, and

(f) optionally repeating any one or a combination of two or more of steps (a)-(e) one or more times to generate heteroalkyl or heterocyclyl metal ion chelating compounds with an improved binding affinity for the membrane protein.

96. A method according to claim 1, wherein the membrane protein is a multidrug resistance protein, e.g. a P-glycoprotein, multidrug resistance associated protein, lung resistance related protein, drug resistance associated protein, breast cancer resistance protein, adenosine triphosphate-binding cassette protein, Bmr, QacA or EmrAB/TolC.

Patentstyret
30 DEC. 1999
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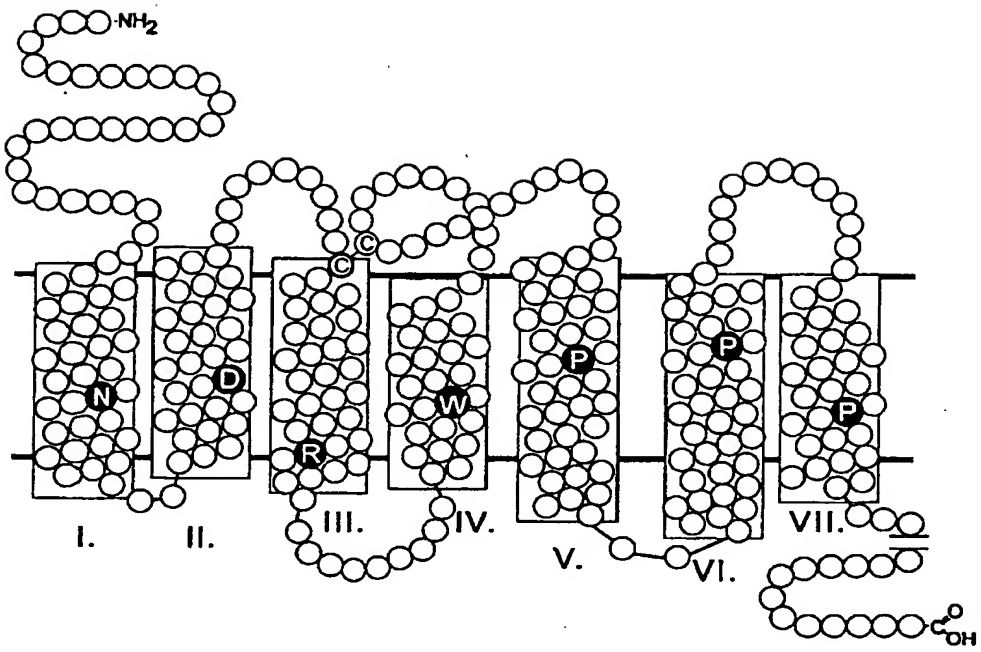

Fig. 1

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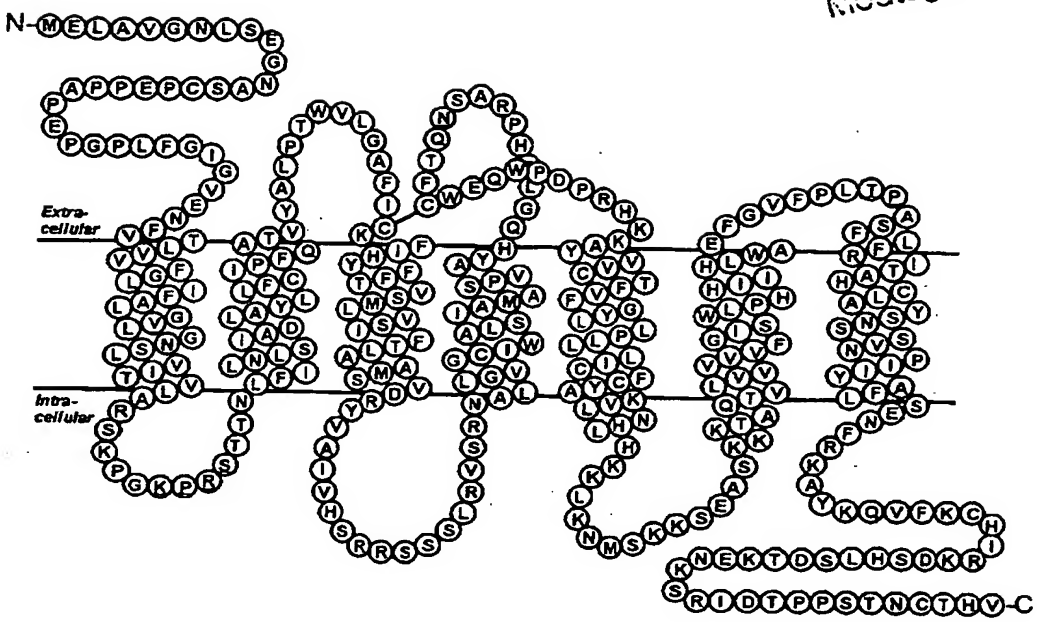

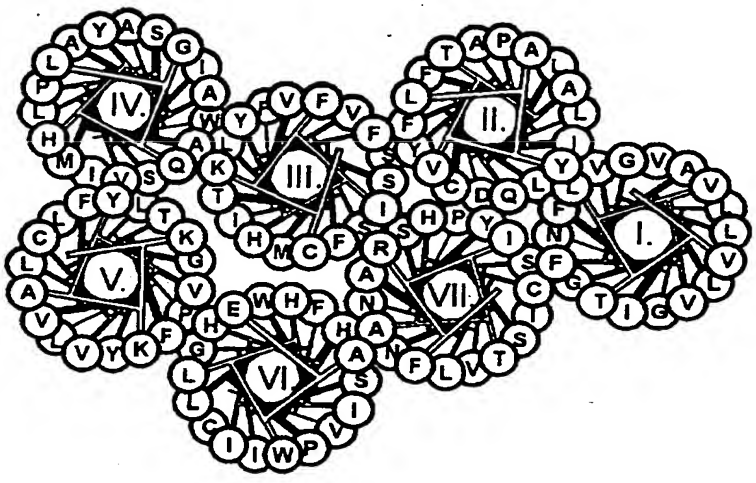

Fig. 2

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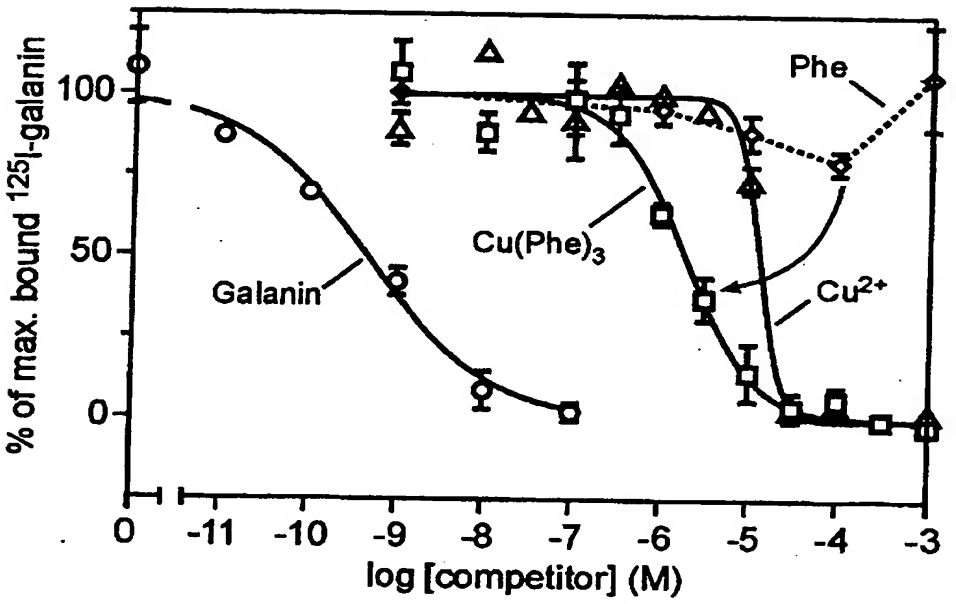

Fig. 3

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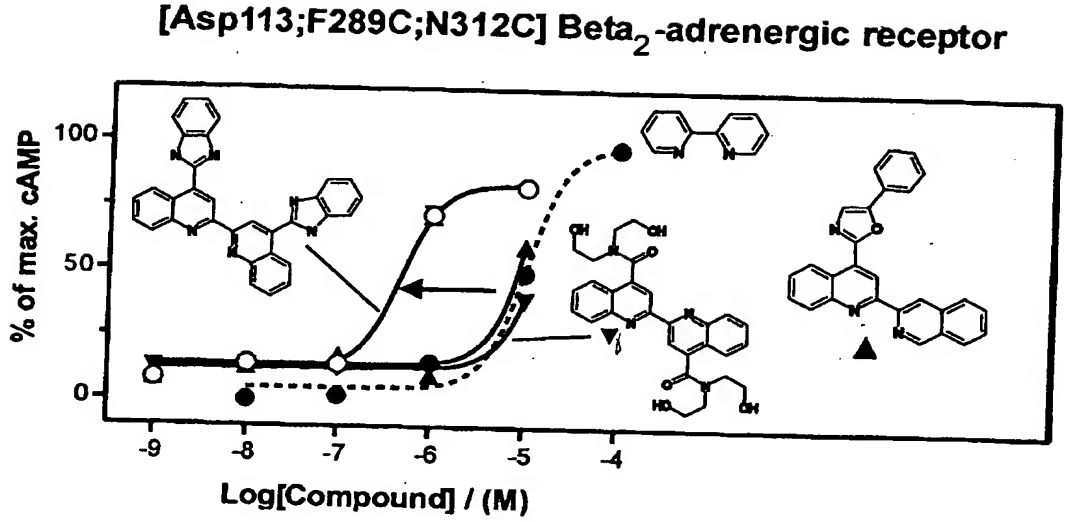

Fig. 4